DESCRIPTION

PLASMID

TECHNICAL FIELD

The present invention relates to a plasmid vector usable in genetic recombinant technology and to a method for expressing a gene by using the plasmid vector. The present invention also relates to a method for isolating a desired gene by using such a plasmid vector. In addition, the present invention relates to a restriction enzyme and a gene thereof available as a genetic engineering reagent, in more detail to the AccIII restriction endonuclease and a DNA coding therefor.

BACKGROUND ART

In constructing an expression system for a desired gene by genetic recombinant technology, expression of the gene is controlled by bringing it under control of a promoter recognized by the RNA polymerase of the host used. In the case of a gene encoding a protein harmful to the host, however, plasmid construction itself is sometimes hampered by expression of the product of the gene due to the inability to stringently control the expression of the promoter used.

As an expression system resolving that problem, the

pET system (produced by Novagen) has been developed, which uses the RNA polymerase of the bacteriophage T7, which infects *Escherichia coli*, with *Escherichia coli* as a host [Journal of Molecular Biology, Vol. 189, pp. 113-130 (1986); Gene, Vol. 56, pp. 125-135 (1987)]. The pET system is a system that allows T7 RNA polymerase, which has high promoter recognition specificity and high transcription activity, to be expressed in *Escherichia coli*, which T7 RNA polymerase transcribes a desired gene placed downstream of the T7 promoter on an expression vector and causes high expression of the gene. Because transcription of the desired gene occurs in the presence of T7 RNA polymerase, plasmid construction in the host is possible without expressing the desired gene, provided that the host does not produce the polymerase; plasmid construction itself is never hampered, as in cases where the expression system is constructed, while the desired gene is kept under control of a promoter recognized by the RNA polymerase of the host.

However, because the T7 RNA polymerase gene has been cloned onto the λ-phage vector and lysogenized into the expression host, there is no freedom of host choice; painstaking procedures are needed if the host is changed. In addition, because the expression of T7 RNA polymerase in the host is not stringently controlled, T7 RNA

polymerase is expressed even when the host is in a non-inductive condition, resulting in expression of the desired gene placed downstream of the T7 promoter on the expression vector even in a non-inductive condition. To suppress such expression of the desired gene in a non-inductive condition, T7 RNA polymerase activity is inhibited using T7 lysozyme, a T7 RNA polymerase inhibitor [Journal of Molecular Biology, Vol. 219, pp. 37-44 (1991)], or T7 RNA polymerase is prevented from getting access to the T7 promoter by placing a lactose operator downstream of the T7 promoter [Journal of Molecular Biology, Vol. 219, pp. 45-59 (1991)].

However, even these countermeasures are unsatisfactory in terms of effect against T7 RNA polymerase of high transcription activity so that the activity of T7 RNA polymerase in a non-inductive condition cannot be completely inhibited. For this reason, if the desired gene product is lethal to the host, it is impossible in some cases to prepare a transformant for expression of the gene, even when plasmid construction is possible. In other words, the pET system involves two problems to be resolved: one of the inability to freely change the host, and the other of inaccurate control of T7 RNA polymerase expression.

On the other hand, there is a bacteriophage having

characteristics similar to those of the bacteriophage T7, known as the bacteriophage SP6 [Science, Vol. 133, pp. 2069-2070 (1961)], which infects Salmonella typhimurium. The RNA polymerase produced by the bacteriophage SP6, a single peptide having a molecular weight of about 100,000, is commonly used for in vitro RNA synthesis since it possesses high promoter recognition specificity and high transcription activity [Journal of Biological Chemistry, Vol. 257, pp. 5772-5778 (1982); Journal of Biological Chemistry, Vol. 257, pp. 5779-5788 (1982)]. In addition, the SP6 RNA polymerase gene has already been cloned and expressed in large amounts in *Escherichia coli* [Nucleic Acids Research, Vol. 15, pp. 2653-2664 (1987)].

Genes whose expression product acts lethally on hosts are exemplified by restriction endonuclease genes. Essentially, restriction endonucleases are utilized for self-defence by cleaving phages and other exogenous DNA entering the cells of microorganisms that produce the restriction endonucleases. On the other hand, microorganisms that produce restriction endonucleases mostly produce modification enzymes that recognize the same base sequences as those of the restriction endonucleases, to protect their own DNA against cleavage by the restriction endonucleases. Specifically, a modification enzyme modifies DNA by adding a methyl group

to one or more bases in the base sequence recognized thereby, to make it impossible for the restriction endonuclease that recognizes the same sequence as that of the modification enzyme to bind thereto or to cleave the DNA. This mechanism is called restriction modification system, and the pair of genes of the restriction endonuclease and modification enzyme that constitute the restriction modification system called restriction modification system gene. Therefore, when the restriction endonuclease gene is expressed in a microorganism lacking a modification enzyme gene from the restriction modification system gene, the microorganism's DNA is cleaved, resulting in cell death. In fact, there are two modification enzyme genes in the MboI restriction modification system gene; it has been reported that cloning of restriction endonuclease genes is impossible due to incomplete modification of the host DNA in the case of incomplete methylation in the co-presence of either modification enzyme gene alone [Nucleic Acids Research, Vol. 21, pp. 2309-2313 (1993)].

Also, it has been demonstrated that if a restriction modification system gene is lost from a cell retaining the restriction modification system gene, a lack of modification activity in the cell results in incomplete methylation of genomic DNA, which in turn causes lethal

cleavage of its own genomic DNA by a very small amount of restriction endonuclease remaining therein [Science, Vol. 267, pp. 897-899 (1995)]. In summary, in the absence of modification enzymes that constitute a restriction modification system, restriction endonucleases behave as proteins very harmful to cells; separate cloning and expression of their genes have been impossible by prior art technologies.

Concerning restriction endonucleases, restriction endonucleases can be classified by their enzymatic properties into three types: I, II and III. Type II restriction endonucleases, in particular, each of which recognizes a particular DNA base sequence and cleaves it at a particular site in or near the sequence, are extensively used in the field of genetic engineering, and restriction endonucleases of this type with various specificities have been isolated from a variety of microorganisms [Nucleic Acids Research, Vol. 24, pp. 223-235 (1996)]. In the present specification, a type II restriction endonuclease is hereinafter referred to as "restriction endonuclease". It should be noted, however, that some microorganisms produce only small amounts of restriction endonuclease, and others produce a plurality of restriction endonucleases. For example, the restriction endonuclease AccIII is produced by

Acinetobacter calcoaceticus (hereinafter referred to as Acc bacterium), which has been deposited under accession number FERM BP-935 at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry [address: 1-3, Higashi 1-chome, Yatabemachi, Tsukuba-gun, Ibaraki, 305, Japan] since November 9, 1985 (date of original deposition), but the amount of the enzyme produced is small and this microorganism also produces the restriction endonucleases AccI and AccII simultaneously. Therefore, advanced production technology is needed to provide the restriction endonuclease AccIII as a reagent of high purity and low cost using this microorganism. In providing a restriction endonuclease as a reagent of high purity and low cost, it is effective to isolate the desired restriction endonuclease gene and selectively produce the desired restriction endonuclease in large amounts by genetic engineering technology. To accomplish this purpose, some methods of isolating restriction endonuclease genes have been reported.

First, there may be mentioned the "shotgun" method, wherein the genomic DNA of a microorganism that produces a restriction endonuclease is cleaved using the appropriate restriction endonuclease, the resulting fragment is inserted into an appropriate plasmid vector, and a clone

expressing the restriction endonuclease gene is selected. Screening methods for desired clones are exemplified by a method wherein a restriction modification system gene is isolated with resistance to phage infection as an index, on the basis of the self-defence function acquired by the host upon introduction of the restriction modification system gene thereinto [PstI: Proceedings of the National Academy of Science of the USA, Vol. 78, pp. 1503-1507 (1981)]. This method, however, necessitates that the size of the restriction modification system gene falls within a range allowing its isolation, and that the expression of the restriction modification system gene isolated exhibits sufficient phage resistance to allow the selective survival of the host. On the other hand, as a general feature of restriction modification system genes, there may be mentioned the close location on the genome of restriction endonuclease genes and modification enzyme genes; in fact, this has been confirmed in many restriction modification system genes that have so far been obtained [Nucleic Acids Research, Vol. 19, pp. 2539-2566 (1991)]. Accordingly, there is a method wherein a restriction modification system gene is screened for with the expression of a modification enzyme gene as an index on the basis of the above-described feature [Japanese Patent Laid-Open No. 63-87982; Nucleic Acids

Research, Vol. 19, pp. 1831-1835 (1991)]. When the restriction endonuclease gene is not close to the modification enzyme gene, however, this method fails to yield the restriction endonuclease gene.

Furthermore, the above-described "shotgun" method poses a fundamental problem associated with a difference in transcription-translation mechanism between the genomic DNA source organism and the host. For example, in the case of insufficient gene expression due to the failure of the promoter and ribosome binding site accompanying the restriction modification system gene to function well in the host, much labor is needed to select transformants containing the desired gene, even if obtained. To avoid this drawback, there is a method wherein the amino acid sequence of the restriction endonuclease protein is analyzed, the restriction endonuclease gene is obtained from the genomic DNA of a microorganism that produces the restriction endonuclease by PCR-based DNA amplification on the basis of the sequence data obtained, and wherein a known protein expression system is utilized [Japanese Patent Laid-Open No. 6-277070]. Because the presence of a restriction endonuclease is lethal to the host in conventional protein expression systems, there is a need to protect the host by, for example, allowing a modification enzyme that constitutes a restriction

modification system together with the enzyme to be co-present.

Although all the above-described methods of the isolation of restriction endonuclease genes necessitate the simultaneous isolation of the restriction endonuclease gene and a modification enzyme gene that constitutes a restriction modification system gene together with the gene, another method of isolating the restriction endonuclease gene alone has been reported [Nucleic Acids Research, Vol. 22, pp. 2399-2403 (1994)]. In that method, however, it is intended to isolate a gene encoding a restriction endonuclease for which optimal temperature for enzyme activity is around 70°C; the co-presence of a modification enzyme gene is necessary when the gene to be isolated encodes a restriction endonuclease showing high specific activity near host culturing temperature.

Exceptionally, there are restriction endonucleases that do not show cleavage activity unless a particular nucleic acid base in their DNA recognition sequence has not been modified by methylation, like the restriction endonuclease DpnI. Genes for restriction endonucleases possessing this property are thought to be exceptional in that they can be isolated even in the absence of another particular gene by selecting the appropriate host organism. In fact, the mrr gene has been isolated, which

encodes the Mrr protein, which is not a type II restriction endonuclease but which recognizes a particular DNA base sequence containing a methylated nucleic acid base and exhibits DNA cleavage activity [Journal of Bacteriology, Vol. 173, pp. 5207-5219 (1991)].

As stated above, isolation of a restriction endonuclease gene by the prior art necessitates the simultaneous expression of the gene and a modification enzyme gene that constitutes a restriction modification system gene together with the gene, except for special cases.

DISCLOSURE OF THE INVENTION

Therefore, a first object of the present invention provides a plasmid vector capable of isolating such a gene that an isolation thereof or a construction of an expression system thereof has been difficult in the prior arts because the gene product is lethal or harmful to a host, and capable of introducing into a host to express the protein efficiently. A second object of the present invention provides a method for expressing a desired gene by using this plasmid vector. A third object of the present invention provides a method for isolating a desired gene by using this plasmid vector, especially for isolating a restriction endonuclease gene without co-

existence of a modification enzyme gene constituting a restriction modification system. A fourth object of the present invention provides a polypeptide possessing an activity of an AccIII restriction endonuclease. In addition, a fifth object of the present invention provides a DNA which encodes a polypeptide possessing an activity of an AccIII restriction endonuclease.

First, to resolve the problems in the pET system, the present inventors have constructed an expression system using SP6 RNA polymerase as a new accurate expression control expression system, and assessed the system.

Because the expression system is constructed using a system plasmid inserted the SP6 RNA polymerase gene into the miniF plasmid, expression systems for the desired gene can be constructed in various strains of *Escherichia coli* by simultaneously introducing an expression vector harboring the desired gene cloned downstream of the SP6 promoter and this system plasmid into the host. In addition, using the lac promoter and antisense technology, the present inventors made it possible to exactly control the expression of the SP6 RNA polymerase gene on the system plasmid. Assessing this expression system using the β-galactosidase gene as a reporter gene demonstrated that the expression of the SP6 RNA polymerase gene is accurately controlled in this system, that there is almost

no expression of SP6 RNA polymerase in a non-inductive condition, and that induction is followed by the expression of a sufficient amount of SP6 RNA polymerase to efficiently express the desired gene and subsequent expression of the desired gene at a high level.

It was also shown, however, that when the host used is *Escherichia coli*, the desired gene downstream of the SP6 promoter is expressed in very small amounts even in a non-inductive condition because the SP6 promoter is very weakly but actually recognized by *Escherichia coli* RNA polymerase. It was thus proven that when the gene product acts very harmfully and lethally to *Escherichia coli*, expression system construction is impossible so that the object of the present invention cannot be accomplished well.

With these findings in mind, the present inventors have made further extensive investigation and unexpectedly found that (i) expression of the desired gene in a non-inductive condition can be suppressed to undetectable levels, and that (ii) expression induction increases the copy number of the plasmid containing the desired gene and causes RNA polymerase expression, resulting in the transcription and translation of the desired gene placed downstream of a promoter recognized by the RNA polymerase, by using a new system for controlling the expression of

the desired gene by means of a combination of two control methods, i.e., control of the copy number of the gene, and transcription control via the promoter. In other words, the plasmid vector of the present invention is a plasmid vector having unique features to resolve the above problems in the field of genetic engineering. The present inventors made further investigation based on this finding, and developed a method for expressing the desired gene using the vector.

The present inventors also have developed a method for isolating a gene whose expression product acts lethally on the host, using the above-described plasmid vector, more specifically a method of isolating a restriction endonuclease gene without the drawbacks of the prior art.

The present inventors also found it possible to isolate DNA encoding the AccIII restriction endonuclease, which had not been obtained so far, without the co-presence of an AccIII modification enzyme, and express the AccIII restriction endonuclease in large amounts, using the above-described method.

(1) The gist of the present invention is concerned with: A plasmid vector characterized by comprising a promoter sequence to control an expression of a desired gene, the promoter sequence being recognized by an RNA polymerase

not inherent to a host, and a replication origin for increasing a copy number by induction with an exogenous factor;

(2) The plasmid vector described in item (1) above, wherein the promoter sequence is recognized by RNA polymerases derived from bacteriophages;

(3) The plasmid vector described in item (2) above, wherein the promoter sequence is recognized by an RNA polymerase derived from SP6 phage;

(4) The plasmid vector described in item (3) above, wherein the promoter sequence contains the base sequence of SEQ ID NO:30 in the Sequence Listing;

(5) The plasmid vector described in any one of items (1) to (4) above, wherein the replication origin is under control of a promoter;

(6) The plasmid vector described in any one of items (1) to (5) above, wherein the replication origin is under control of the lac promoter;

(7) The plasmid vector described in any one of items (1) to (6) above, comprising a drug resistance gene as a selection marker;

(8) The plasmid vector described in item (7) above, which is selected from pACE601, pACE611, pACE701 and pACE702;

(9) A plasmid vector in which a desired gene to be expressed is incorporated into the plasmid vector

described in any one of items (1) to (8) above;

(10) A method for expressing a desired gene, characterized by introducing into a host a plasmid vector in which the desired gene is incorporated into the plasmid vector described in any one of items (1) to (8) above, and an RNA polymerase gene which recognizes a promoter sequence in the plasmid vector, and inducing an increase in a copy number of the plasmid vector and an expression of the RNA polymerase by using an exogenous factor to transcribe and translate the desired gene;

(11) The method for expressing a desired gene described in item (10) above, characterized in that the increase in the copy number of the plasmid vector and the expression of the RNA polymerase are induced by respective exogenous factors;

(12) The method for expressing a desired gene described in item (10) above, characterized in that the increase in the copy number of the plasmid vector and the expression of the RNA polymerase are induced by a same exogenous factor;

(13) The method for expressing a desired gene described in any one of items (10) to (12) above, wherein the exogenous factor which induces the increase in the copy number of the plasmid vector, is one or more selected from the group consisting of an addition of isopropyl-β-D-thiogalactoside

(IPTG), an addition of lactose, an addition of galactose, an addition of arabinose, a reduction of a tryptophane concentration and an adjustment of a transformant cultivation temperature;

(14) The method for expressing the desired gene described in any one of items (10) to (12) above, wherein the exogenous factor which induces the expression of the RNA polymerase, is one or more selected from the group consisting of an addition of isopropyl-β-D-thiogalactoside (IPTG), an addition of lactose, an addition of galactose, an addition of arabinose, a reduction of a tryptophane concentration and an adjustment of a transformant cultivation temperature;

(15) The method for expressing a desired gene described in item (10) above, characterized in that the RNA polymerase gene is introduced into the host by the other plasmid vector or a phage vector;

(16) The method for expressing a desired gene described in item (10) above, characterized in that the RNA polymerase gene is incorporated into a chromosome of the host;

(17) The method for expressing a desired gene described in item (15) or item (16) above, characterized in that the RNA polymerase gene is derived from SP6 phage;

(18) The method for expressing a desired gene described in any one of items (10) to (17) above, wherein the desired

gene encodes a protein lethal or harmful to the host;

(19) The method for expressing a desired gene described in any one of items (10) to (18) above, characterized in that *Escherichia coli* is used as the host;

(20) A method for isolating a desired gene, characterized in that the plasmid vector described in any one of items (1) to (8) above is employed in the method for isolating the desired gene;

(21) The method for isolating a desired gene described in item (20) above, wherein the desired gene encodes a protein lethal or harmful to a host;

(22) The method for isolating a desired gene described in item (21) above, wherein the gene encoding a protein lethal or harmful to the host is a restriction endonuclease gene;

(23) A polypeptide containing the entire or a portion of the amino acid sequence shown by SEQ ID NO:1 in the Sequence Listing, and possessing an activity of AccIII restriction endonuclease;

(24) A polypeptide having an amino acid sequence resulting from at least one of deletion, addition, insertion or substitution of one or more amino acid residues in the amino acid sequence of SEQ ID NO:1 in the Sequence Listing or a portion thereof, and possessing an activity of AccIII restriction endonuclease;

(25) A DNA encoding a polypeptide which contains the entire or a portion of the amino acid sequence shown by SEQ ID NO:1 in the Sequence Listing, and possesses an activity of AccIII restriction endonuclease;

(26) A DNA containing the entire or a portion of the DNA shown by SEQ ID NO:2 in the Sequence Listing wherein an expression product of the DNA possesses an activity of AccIII restriction endonuclease;

(27) A DNA encoding a polypeptide resulting from at least one of deletion, addition, insertion or substitution of one or more amino acid residues in the amino acid sequence of SEQ ID NO:1 in the Sequence Listing or a portion thereof, and possessing an activity of AccIII restriction endonuclease; and

(28) A DNA capable of hybridizing to the DNA described in any one of items (25) to (27) above, and encoding a polypeptide possessing an activity of AccIII restriction endonuclease.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the constitution of the model expression plasmids pMSP6L and pMSP6F.

Figure 2 shows the constitution of the model expression plasmid pMSP60.

Figures 3 (A), (B) show the procedure of the

construction of the runaway plasmid pHS2870.

Figure 4 shows the constitution of the runaway plasmid pHS2870.

Figures 5 (A), (B) show the procedure of the construction of the plasmid pCRS04.

Figure 6 shows the constitution of the plasmid pCRS04.

Figure 7 shows the constitution of the plasmid pCRA19.

Figures 8 (A), (B), (C), (D), (E) show the procedure of the construction of the system plasmid pFSP6.

Figures 9 (A), (B) show the procedure of the construction of the plasmid pACE601.

Figure 10 shows the constitution of the plasmid pACE611.

Figures 11 (A), (B) show the procedure of the construction of the plasmid pACE611.

Figures 12 (A), (B) show the procedure of the construction of the plasmid pCRS70.

Figures 13 (A), (B) show the procedure of the construction of the plasmids pACE701 and pACE702.

Figure 14 shows the expression of the activity of Nsp 7524 III restriction endonuclease depending on the induction or non-induction of IPTG, by agarose gel electrophoresis of the degradation reaction solution of λ-

DNA after introducing pFSP6 and pACE601 or pFSP6 and pACE601-NspIII into *Escherichia coli* HB101. In this figure, M indicates the λ-EcoT141 size marker; 1 indicates AvaI digested λ-DNA; 2 indicates HB101/pFSP6/pACE601 after induction; 3 indicates HB101/pFSP6/pACE601 before induction; 4 indicates HB101/pFSP6/pACE601-NspIII after induction; and 5 indicates HB101/pFSP6/pACE601-NspIII before induction.

Figure 15 shows a result of an agarose gel electrophoresis in the case of 2 and 3 hour-elongation of the degradation reaction of λ-DNA depending on the induction or non-induction of IPTG after introducing pFSP6 and pACE601 or pFSP6 and pACE601-NspIII into *Escherichia coli* HB101. In this figure, M indicates the λ-EcoT141 size marker; 1 indicates AvaI digested λ-DNA; 2 indicates HB101/pFSP6/pACE601 after 1 hour (induction); 3 indicates HB101/pFSP6/pACE601 after 2 hours (induction); 4 indicates HB101/pFSP6/pACE601 after 3 hours (induction); 5 indicates HB101/pFSP6/pACE601 after 1 hour (non-induction); 6 indicates HB101/pFSP6/pACE601 after 2 hours (non-induction); 7 indicates HB101/pFSP6/pACE601 after 3 hours (non-induction); 8 indicates HB101/pFSP6/pACE601-NspIII after 1 hour (induction); 9 indicates HB101/pFSP6/pACE601-NspIII after 2 hours (induction); 10 indicates HB101/pFSP6/pACE601-NspIII after 3 hours (induction); 11

indicates HB101/pFSP6/pACE601-NspIII after 1 hour (non-induction); 12 indicates HB101/pFSP6/pACE601-NspIII after 2 hours (non-induction); and 13 indicates HB101/pFSP6/pACE601-NspIII after 3 hours (non-induction).

Figure 16 shows the construction of the AccIII restriction modification system gene.

BEST MODE FOR CARRYING OUT THE INVENTION

The first aspect of the present invention relates to a plasmid vector. More specifically, it relates to a plasmid vector characterized in that the plasmid vector comprises a promoter sequence, which is recognized by an RNA polymerase not inherent to the host, to control the expression of the desired gene, and a replication origin for increasing the copy number by induction with an exogenous factor. The promoter sequence contained in the plasmid vector, which sequence controls the expression of the desired gene, may be any promoter sequence; promoter sequences recognized by particular RNA polymerases, e.g., those recognized by RNA polymerases derived from the T7, T3, SP6 and other bacteriophages, can be used, with preference given to those recognized by the RNA polymerase derived from the SP6 phage. The base sequence of the minimum region of the promoter recognized by the RNA polymerase derived from the SP6 phage, i.e., the SP6

promoter, is shown by SEQ ID NO:30 in the Sequence Listing. Furthermore, the plasmid vector may have a drug resistance gene used as a selection marker.

Exogenous factors that control the copy number of the plasmid vector of the present invention, i.e., the function of the replication origin of the plasmid vector, include, but are not limited to, for example, the addition of various chemicals, such as lactose or structurally related substances like isopropyl-β-D-thiogalactoside (IPTG), galactose or structurally related substances, and arabinose or structurally related substances; reduction of tryptophane concentration; and adjustment of transformant cultivation temperature. By bringing the replication origin under control of a promoter that can be controlled by these factors, copy number control becomes possible. Promoters usable for this purpose include, but are not limited to, the lac, trp, tac, gal, ara and P_L promoters etc. when the host used is *Escherichia coli*, as long as the above-described purpose is accomplished. These promoters can also be used to induce the expression of an RNA polymerase gene.

Examples of the replication origin of the plasmid vector of the present invention include, but are not limited to, plasmid vectors that serve as replication origins of runaway plasmids under control of the lac

promoter. In this case, copy number control is achieved by the addition of lactose and structurally related substances, most preferably by the addition of isopropyl-β-D-thiogalactoside (IPTG).

The second aspect of the present invention relates to a method for expressing a desired gene to be expressed, characterized by introducing a plasmid vector prepared by incorporating the desired gene into the plasmid vector of the present invention, and an RNA polymerase gene that recognizes the promoter sequence on the plasmid vector, into a host, and inducing an increase in the copy number of the plasmid vector and the expression of the RNA polymerase with an exogenous factor to transcribe and translate the desired gene. RNA polymerase genes that recognize the promoter sequence on the plasmid vector include, but are not limited to, for example, the RNA polymerase genes derived from the above-mentioned bacteriophages, with preference given to the RNA polymerase gene derived from the SP6 phage. Exogenous factors that induce the expression of such RNA polymerase or an increase in the copy number of the plasmid vector include, but are not limited to, for example, the addition of various chemicals, such as lactose or structurally related substances like isopropyl-β-D-thiogalactoside (IPTG), galactose or structurally related substances, and

arabinose or structurally related substances; the reduction of tryptophane concentration; and the adjustment of transformant cultivation temperature.

Induction of an increase in the copy number of plasmid vector and that of expression of RNA polymerase may be achieved by the action of respective exogenous factors or a same exogenous factor. Also, in this case, the gene encoding RNA polymerase may be incorporated onto a chromosome of the host, or introduced into the host by a plasmid vector other than the plasmid vector of the present invention or a phage vector. In the latter case, the host can readily be changed according to the purpose. In that case, introduction of the vector having an RNA polymerase gene into the host may precede or follow the introduction of the plasmid vector of the present invention.

Although the desired gene in the present invention is not subject to limitation, it is meaningful when it encodes a protein lethal or harmful to the host. Such proteins include, for example, the above-mentioned restriction endonucleases, other nucleases, nucleic acid-binding proteins, and proteases.

The present invention provides a system that controls the expression of a desired gene by a combination of two control methods, i.e., control of the copy number of the

gene, and control of transcription via a promoter, as described above. Control of the expression of the desired gene cloned onto a plasmid in the present invention, unlike conventional control, is very stringent so that its expression can be suppressed to an undetectable level in a non-inductive condition. When the copy number of the plasmid, i.e., the copy number of the desired gene, is increased, with concurrent expression of RNA polymerase, by expression induction, the desired gene is transcribed and translated under control of the promoter by the action of the RNA polymerase.

It is therefore possible to use the plasmid vector of the present invention to prepare a transformant containing a gene harmful or lethal to the host and express the gene, a task difficult to achieve by the prior art.

For example, when cloning of a gene encoding the Nsp7524 III restriction endonuclease was attempted using the plasmid vector of the present invention, the gene was successfully retained in *Escherichia coli* without co-presence of the corresponding modification enzyme gene. It was also possible to express the Nsp7524 III restriction endonuclease in the cells of the resulting transformant by introducing a system plasmid containing an RNA polymerase gene into the transformant to induce the expression of the Nsp7524 III restriction endonuclease.

Also, using the plasmid vector of the present invention as a cloning vector for preparation of a gene library, it is possible to isolate a gene that cannot be isolated by a conventional method, and confirm the activity of its expression product in a single host. The use of the plasmid vector of the present invention is of course not limited to the cloning of a gene encoding a product harmful to the host, and can be used as a general-purpose plasmid vector.

An RNA polymerase gene relating to the present invention can be introduced into a host using, for example, the plasmid pFSP6 and the phage M13sp6. Details of construction of the plasmid pFSP6 are shown in Reference Example (2). *Escherichia coli* HB101 as transformed with the plasmid, designated *Escherichia coli* HB101/pFSP6, has been deposited under accession number FERM BP-5742 at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry [address: 1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, 305, Japan] since December 22, 1995 (date of original deposition). A method for the construction of the phage M13sp6 is described in Example 2 (6).

The present invention is hereinafter described in more detail with reference to a case using the plasmid

pFSP6.

First described are the results of an experiment conducted using the constructed multicopy model expression plasmids pMSP6L, pMSP6F (Figure 1) and pMSP6O (Figure 2), all having a promoter sequence recognized by SP6 RNA polymerase and the β-galactosidase gene as a reporter gene, and all allowing the expression of the desired gene using the above-described system plasmid pFSP6.

Details of construction of these model expression plasmids are shown in Reference Example (3). These plasmids incorporate the minimum region of the SP6 promoter (pMSP6L), a inherent SP6 promoter region (pMSP6F), or both the minimum region of the SP6 promoter and the lac operator region (pMSP6O), respectively, as an SP6 plasmid sequence.

When the plasmid pMSP6L or pMSP6F alone was introduced into the *Escherichia coli* strains shown in Table 1, β-galactosidase activity was noted, despite the fact that the host *Escherichia coli* did not express SP6 RNA polymerase, the amounts being higher than that obtained with the plasmid pMS434, which does not have the SP6 promotor, as shown in Table 2. In short, it can be conjectured that the SP6 promoter is recognized by the RNA polymerase derived from *Escherichia coli*, and that the β-galactosidase gene downstream thereof is transcribed and

translated. Also, when *Escherichia coli* MRi80, a strain that has a mutation in the pcnB gene and wherein the copy number of a plasmid having a replication origin derived from ColE1, like the above-described model of expression plasmids, is reduced by 1/6 to 1/13 of that of the parent strain MRi7, was used as a host, the β-galactosidase activity was also decreased to 1/7 to 1/14 of that of MRi7. This suggests a correlation between the β-galactosidase gene expression level and the copy number of the plasmid introduced.

Table 1

Strain	Genotype	Origin / Reference
MC4100	F-, araD139, Δ(argF-lac)U169, thiA, rpsL150, relA1, flbB5301, deoC1, ptsF25, rbsR	Casadaban et al. J. Mol. Biol. 104(1976) 541-555.
MRi7	MC4100 Δ rbs-7	Lopilato et al. J. Bacteriol. 158(1984) 665-673
MRi80	MRi7 pcnB80	Lopilato et al. Mol. Gen. Genet. 205(1986) 285-290.

Table 2

Strain	β − Galactosidase Activity		
	Plasmid		
	pMSP6F	pMSP6L	pMS434
MC4100	202	242	30
MRi7	234	287	23
MRi80	17	27	4

Next, when the same model of expression plasmids were introduced into *Escherichia coli* together with the above-described system plasmid pFSP6 and β-galactosidase activity was determined under conditions such that the expression of the SP6 RNA polymerase gene was not induced, the β-galactosidase activity was at most as high as that obtained with *Escherichia coli* not containing the system plasmid, as shown in Table 3. This demonstrates that the expression of the SP6 RNA polymerase gene on the plasmid pFSP6 is almost completely suppressed in a non-inductive condition. On the other hand, when the expression of the SP6 RNA polymerase gene was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG), β-galactosidase activity was increased by 18 to 32 times, demonstrating that the β-galactosidase gene can be expressed via the SP6 promoter. When the host used was *Escherichia coli* MRi80, in particular, β-galactosidase activity was increased to a level 25 to 32 times that obtained in a non-inductive condition, 4 hours after induction, though the activity in a non-inductive condition remained at a very low level.

Table 3

Strain	β − Galactosidase Activity (Non-Induction/Induction)					
	pMSP6F		pMSP6L		pMS434	
	Non-Induction	Induction	Non-Induction	Induction	Non-Induction	Induction
MC4100(pFSP6)	221	4875	234	5256	25	19
MRi7(pFSP6)	220	3958	242	4243	25	21
MRi80(pFSP6)	17	425	30	970	3	ND

In the plasmid pMSP60, which carries the lac operator sequence downstream of the SP6 promoter, the β-galactosidase activity under non-inductive conditions is lower than that in other model expression plasmids; however, its expression cannot be suppressed completely. These results suggest that the expression of the desired gene is difficult to completely suppress in an expression system using a multicopy plasmid, and that controlling the copy number of the plasmid containing the desired gene is more effective in resolving this problem than controlling the expression of the RNA polymerase gene on the system plasmid.

The present inventors thus made further investigation based on these results to explore a new expression vector, and developed the plasmid vector of the present invention.

Specifically, the present inventors constructed a runaway plasmid vector capable of reducing the expression level in a non-inductive condition to ensure sufficient expression after induction. First, in consideration of the fact that SP6 RNA polymerase expression in a system plasmid is induced by IPTG, a plasmid having a runaway replication origin allowing an increase in copy number by IPTG induction was constructed in accordance with the procedures shown in Figure 3. First, the plasmid pUC106AdPO is constructed by removing the PvuII fragment containing the lac promoter and the operator region from the plasmid pUC106A, which is obtained by introducing the 106 NdeI DNA fragment prepared from the two DNA strands whose base sequences are shown by SEQ ID NO:3 and SEQ ID NO:4 in the Sequence Listing into the NdeI site of the plasmid vector pUC19 (produced by Takara Shuzo).

Next, the plasmid pHS2870 can be obtained by inserting the RNA1870 fragment obtained by PCR using the RNAIIA primer (base sequence shown by SEQ ID NO:5 in the Sequence Listing) arranged near the RNAII region (replication origin of the plasmid) and the 1870 primer (base sequence shown by SEQ ID NO:6 in the Sequence Listing) arranged at a terminus of the 106 NdeI sequence with the plasmid as a template, into the XbaI site of the plasmid pSTV28 (produced by Takara Shuzo). The plasmid

pHS2870 thus constructed is shown in Figure 4. Generally, the plasmid is present at a copy number of about 30 copies per *Escherichia coli* cell; however, this copy number is increased to several hundreds by the addition of IPTG.

The AccI-NspI fragment containing the P15A replication origin derived from the plasmid pSTV28 is then removed from the plasmid (plasmid pCRS01), followed by further removal of the EcoRI-XbaI fragment containing an unnecessary restriction endonuclease site (plasmid pCRS02), after which a DNA fragment containing the lactose repressor (lacIq) gene derived from the plasmid pMJR1560 [Gene, Vol. 51, pp. 225-267 (1987)] is introduced to yield the plasmid pCRS04. A flow diagram of the construction of the plasmid pCRS04 from the plasmid pHS2870 is shown in Figure 5. The plasmid pCRS04 thus constructed is shown in Figure 6. The plasmid is a runaway plasmid induced by IPTG; generally, the plasmid is present at a copy number of 1 to 2 copies per *Escherichia coli* cell; however, this copy number is increased to several hundreds by the addition of IPTG.

To control the expression of the desired gene inserted into the plasmid, the P_{SP6}-O_{lac} EX linker, a double-stranded oligonucleotide containing the SP6 promoter sequence and lac operator sequence, may be introduced into the NheI site of the plasmid to construct

the plasmid pACE601. The P_{SP6}-O_{lac} EX linker is prepared from the two DNA strands whose base sequences are shown by SEQ ID NO:7 and SEQ ID NO:8 in the Sequence Listing. The plasmid pACE601 is a runaway plasmid vector having the chloramphenicol resistance gene as a selection marker.

Also, by introducing the BspHI fragment containing the β-lactamase gene derived from the plasmid vector pUC118 (produced by Takara Shuzo) into the NheI site of the above-described plasmid pCRS04 (plasmid pCRS70), subsequently removing the NcoI-BsaAI fragment containing the chloramphenicol resistance gene, and replacing it with the above-described P_{SP6}-O_{lac} EX linker, the plasmids pACE701 and pACE702 can be constructed, which plasmids have the linker inserted in mutually opposite directions. These plasmids are runaway plasmid vectors having the ampicillin resistance gene as a selection marker.

The potential of the thus-obtained plasmids pACE601, pACE701 and pACE702 used for control of the expression of the desired gene can be determined using the β-galactosidase gene as a reporter gene, as described above. It is possible to construct model expression plasmids by introducing a DNA fragment containing the β-galactosidase gene as amplified by PCR using the primers trpA-N-NcoI and lacZ-C-NcoI with the plasmid pMS434 [Gene, Vol. 57, pp. 89-99 (1987)] as a template, into the NcoI

site downstream of the SP6 promoter in each of the above-described three plasmids. The base sequences of the primers trpA-N-NcoI and lacZ-C-NcoI are shown by SEQ ID NO:9 and SEQ ID NO:10 in the Sequence Listing, respectively. The plasmids thus obtained are designated pACE601Z, pACE701Z and pACE702Z, respectively. In *Escherichia coli* transformed with these model expression plasmids, absolutely no β-galactosidase activity is detected, demonstrating very exact control of the expression of the β-galactosidase gene.

Using the Nsp7524 III restriction endonuclease gene, it is possible to confirm that a gene whose expression product acts lethally on the host can be isolated and expressed using the plasmid of the present invention. The plasmid pBRN3 contains the Nsp7524 III restriction modification system gene. *Escherichia coli* MC1061 as transformed with the plasmid, designated *Escherichia coli* MC1061/pBRN3, has been deposited under accession number FERM BP-5741 at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry [address: 1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, 305, Japan] since September 28, 1995 (date of original deposition). By a PCR-based DNA amplification reaction using a pair of the primers L-ORF and NspR-ORF3 with the

plasmid pBRN3 prepared from the transformant as a template, a DNA fragment containing the Nsp7524 III restriction endonuclease gene alone can be obtained. The base sequences of the primers L-ORF and NspR-ORF3 are shown by SEQ ID NO:11 and SEQ ID NO:12 in the Sequence Listing, respectively. By introducing into *Escherichia coli* HB101 the plasmid pACE601-NspIII, which is obtained by inserting the resulting DNA fragment into the NcoI site downstream of the SP6 promoter of the above-described plasmid pACE601, the transformant *Escherichia coli* HB101/pACE601-NspIII can be obtained. The transformant allows the host to stably retain the Nsp7524 III restriction endonuclease gene despite the absence of the Nsp7524 III modification enzyme gene.

Furthermore, the transformant *Escherichia coli* HB101/pFSP6/pACE601-NspIII can be prepared by introducing the above-described system plasmid pFSP6 into the transformant. By culturing the transformant and inducing expression by the addition of IPTG at the appropriate time, the possibility of production of the Nsp7524 III restriction endonuclease in the culture can be confirmed.

The third aspect of the present invention provides a method for isolating a desired gene characterized by using the above-described plasmid vector. The isolation method of the present invention is suitably applied to the

isolation of a gene whose expression product is lethal or harmful against the host, especially a restriction endonuclease gene. By using the present method, it is possible to isolate a restriction endonuclease gene that has not been isolated so far, e.g., the AccIII restriction endonuclease gene, even in the absence of the AccIII modification enzyme gene. Isolation of a restriction endonuclease gene can be achieved not only by the "shotgun method", wherein the genomic DNA of a microorganism that produces the desired enzyme is cleaved with the appropriate restriction endonuclease, and the resulting DNA fragment is inserted directly into, for example, the plasmid pACE611, but also by the use of a cassette library of the genomic DNA of a microorganism that produces the restriction endonuclease. By obtaining a gene by DNA amplification with the cassette library as a template, it is possible to express a gene prepared from a microorganism whose transcription mechanism differs from that of the host. The method of isolating a restriction endonuclease gene using a cassette library is hereinafter described with reference to an example involving the AccIII restriction endonuclease.

A cassette library of the genomic DNA of a microorganism that produces the AccIII restriction endonuclease, i.e., an Acc bacterium, can be prepared as

follows: Genomic DNA is extracted from the cell culture of an Acc bacterium, and digested with the appropriate restriction endonuclease, after which the resulting DNA fragment is ligated to a cassette having a protruding end complementary to the fragment. Several similar cassette libraries are prepared using different restriction endonucleases for genomic DNA cleavage. These libraries are generically referred to as an Acc genomic cassette library. Next, the desired restriction endonuclease protein is purified from the Acc bacterium, its amino acid sequence is partially determined, and a primer is synthesized on the basis of the sequence. Using this primer and cassette primer, a PCR-based DNA amplification reaction is carried out with each cassette library as a template, to obtain a DNA fragment containing an AccIII restriction endonuclease gene fragment. The base sequence of the DNA fragment obtained is determined by, for example, direct sequencing of the PCR product, to determine the full-length base sequence of the AccIII restriction endonuclease gene.

By designing a primer capable of amplifying the full-length sequence of the AccIII restriction endonuclease gene from the base sequence, and carrying out a PCR-based DNA amplification reaction using this primer with the genomic DNA of the Acc bacterium as a template, a

DNA fragment containing the full-length sequence of the AccIII restriction endonuclease gene is obtained. The DNA fragment obtained is inserted downstream of the SP6 promoter of the plasmid pACE611 so that the codon frames are adjusted, and the resulting recombinant plasmid is introduced into, for example, *Escherichia coli* JM109, to yield transformants. Transformants containing the AccIII restriction endonuclease gene in an expressible condition can be selected by culturing each transformant, inducing gene expression by the gene expression method of the present invention, and determining the AccIII restriction endonuclease activity in each culture obtained, as well as by drawing the restriction endonuclease map of the plasmid DNA harbored by each transformant.

The AccIII restriction endonuclease gene thus obtained was actually inserted into the plasmid pACE611, and the resulting plasmid was designated pCRA19. The restriction endonuclease map of this plasmid is shown in Figure 7, wherein the bold solid line indicates the DNA fragment containing the AccIII restriction endonuclease gene. *Escherichia coli* JM109 as incorporating the plasmid pCRA19, designated *Escherichia coli* JM109/pCRA19, has been deposited under accession number FERM BP-5743 at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of

International Trade and Industry [address: 1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, 305, Japan] since May 28, 1996 (date of original deposition). The transformant stably retains the restriction endonuclease gene, despite the absence of the AccIII modification enzyme gene therein. By the above-described method, a restriction endonuclease gene can be isolated without the co-presence of a modification enzyme gene that constitutes a restriction modification system gene.

The fourth aspect of the present invention provides a polypeptide possessing an activity of the AccIII restriction endonuclease.

In the present specification, the term "AccIII restriction endonuclease" as a general term of polypeptides possessing an activity of the AccIII restriction endonuclease may be used in some cases. The AccIII restriction endonuclease of the present invention comprises the amino acid sequence described in SEQ ID NO:1 in the Sequence Listing, for example.

The AccIII restriction endonuclease of the present invention also includes, but not limited to the above, the polypeptide containing the entire or a portion of the amino acid sequence described in SEQ ID NO:1 in the Sequence Listing and possessing an activity of the AccIII restriction endonuclease. Furthermore, the polypeptide

having an amino acid sequence resulting from at least one of deletion, addition, insertion or substitution of one or more amino acid residues in the amino acid sequence of SEQ ID NO:1 or a portion thereof in the Sequence Listing and possessing an activity of the AccIII restriction endonuclease is included in the scope of the present invention.

Generally, a naturally-occurring protein can undergo deletion, insertion, addition, substitution and other variations of amino acid residues in its amino acid sequence due to modifications, etc. of the protein in vivo or during purification, as well as those due to polymorphism and variation of the gene encoding it. Nevertheless, it is known that there are some such polypeptides which are substantially equivalent to variation-free proteins in terms of physiological and biological activity. Thus, those structurally different from the corresponding protein, but having no significant difference of function or activity from the protein is within the scope of the present invention. It is also the same when artificially introducing the above variations to the amino acid sequence of a protein and in this case, it is possible to produce more diverse variants. For example, the methionine residue at the N-terminus of a

protein expressed in *Escherichia coli* is reportedly often removed by the action of methionine aminopeptidase, but the removal is not completely done depending on the kinds of proteins, and some such expressed proteins have the methionine residue and others not. However, the presence or absence of the methionine residue does not affect protein activity in most cases. It is also known that a polypeptide resulting from replacement of a particular cysteine residue with serine in the amino acid sequence of human interleukin 2 (IL-2) retains IL-2 activity [Science, 224, 1431 (1984)].

In addition, in producing a protein by gene engineering, the desired protein is often expressed as a fused protein. For example, the N-terminal peptide chain derived from another protein is added to the N-terminus of the desired protein to enhance the expression of the desired protein, or purification of the desired protein is facilitated by adding an appropriate peptide chain to the N- or C-terminus of the desired protein, expressing the protein, and using a carrier showing affinity for the peptide chain added. Thus, even if the polypeptide has an amino acid sequence partially different from the AccIII restriction endonuclease of the present invention, it is within the scope of the present invention as long as it possesses essentially equivalent activity to the AccIII

restriction endonuclease of the present invention.

The AccIII restriction endonuclease can be obtained by, for example, culturing the above-described transformant *Escherichia coli* JM109/pCRA19, which contains the AccIII restriction endonuclease gene, adding the inducing agent IPTG at an appropriate time during its cultivation to increase the copy number of pCRA19, and subsequently infecting with the phage M13sp6.

The AccIII restriction endonuclease can be harvested from the transformant culture by, for example, collecting cells from the culture, subsequently extracting the enzyme by ultrasonic disruption, ultracentrifugation, etc., and then purifying the enzyme by a combination of nucleic acid removal, salting-out, affinity chromatography, gel filtration, ion exchange chromatography, etc. Because the above-described culture, which serves as the starting material for this purification, does not contain other restriction endonucleases, such as AccI and AccII, an enzyme preparation of desired purity can be obtained more easily than by conventional purification methods.

The fifth aspect of the present invention provides a DNA encoding a polypeptide possessing an activity of the AccIII restriction endonuclease.

The DNA of the present invention encoding a polypeptide possessing an activity of the AccIII

restriction endonuclease comprises a DNA encoding the amino acid sequence described in SEQ ID NO:1 in the Sequence Listing, and includes but not limited to, a DNA comprising the base sequence described in SEQ ID NO:2 in the Sequence Listing, for example. Specifically, the following DNAs are within the scope of the present invention.

(1) a DNA encoding a polypeptide which contains the entire or a portion of the amino acid sequence described in SEQ ID NO:1 in the Sequence Listing and possesses an activity of the AccIII restriction endonuclease;

(2) a DNA containing the entire or a portion of the DNA shown in SEQ ID NO:2 in the Sequence Listing, wherein the expression product of the DNA possesses an activity of the AccIII restriction endonuclease;

(3) a DNA encoding a polypeptide resulting from at least one of deletion, addition, insertion or substitution of one or more amino acid residues in the amino acid sequence of SEQ ID NO:1 in the Sequence Listing or a portion thereof and possessing an activity of the AccIII restriction endonuclease; and

(4) a DNA capable of hybridizing to the DNA described in above (1) to (3), and encoding a polypeptide possessing an activity of the AccIII restriction endonuclease, etc..

In addition, if the hybridization is carried out

under the stringent condition using the above obtained DNA as a probe, a similar DNA somewhat different from the obtained DNA (SEQ ID NO:2 in the Sequence Listing) but encoding a polypeptide possessing the same enzyme activity, can be obtained. Such a DNA is also included in the scope of the present invention.

Such a stringent condition refers to that the membrane with DNA immobilized thereon is subjected to hybridization with the probe in a solution containing 6 × SSC (1 × SSC is a solution of 8.76 g of NaCl and 4.41 g of sodium citrate in 1 liter of water), 1% SDS, 100 μg/ml salmon sperm DNA, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone and 0.1% Ficoll, incubating at 65°C for 20 hours, for example.

Methods for obtaining similar DNA encoding the AccIII restriction endonuclease by hybridization include, for example, the following method.

First, DNA obtained from an appropriate gene source is ligated to a plasmid or a phage vector by a conventional method to yield a DNA library. This library is introduced into an appropriate host; the resulting transformants are cultured on plates; colonies or plaques that have grown are transferred onto nitrocellulose or nylon membranes and denatured, after which the DNA is

fixed onto the membrane. These membranes are incubated for hybridization in a solution of the above-described composition containing a probe previously labeled with ^{32}P etc. (probe used may be any polynucleotide encoding the entire or a portion of the amino acid sequence shown by SEQ ID NO:1 in the Sequence Listing, exemplified by a polynucleotide consisting of, or containing, the entire or a portion of the base sequence shown by SEQ ID NO:2 in the Sequence Listing) under the conditions shown above. After completion of the hybridization, the non-specifically adsorbed probe is washed out, followed by autoradiography etc., to identify clones that have hybridized to the probe. This procedure is repeated until the desired hybridizing clone is isolated. The clone thus obtained retains DNA encoding a polypeptide having the desired enzyme activity.

The DNA obtained is determined for base sequence to confirm that it encodes the desired enzyme protein, by, for example, the method described below.

For base sequencing, the transformant is cultured in a test tube etc. when the host is *Escherichia coli*, and a plasmid is prepared by a conventional method, provided that the transformant has been prepared using a plasmid vector. Using the plasmid obtained as a template as is, or after the insert is taken out and subcloned into the

M13 phage vector etc., the base sequence is determined by the dideoxy method. In the case of a transformant prepared using a phage vector as well, the base sequence can be determined by basically the same procedures.

These basic experimental processes from cultivation to base sequencing are described in, for example, Molecular Cloning: A Laboratory Manual, 1982, T. Maniatis et al., published by Cold Spring Harbor Laboratory.

Whether or not the DNA obtained is similar DNA encoding the desired AccIII restriction endonuclease can be confirmed by comparing the determined base sequence with the base sequence shown by SEQ ID NO:2 in the Sequence Listing, or by comparing the amino acid sequence deduced from the determined base sequence with the amino acid sequence shown by SEQ ID NO:1 in the Sequence Listing.

When the DNA obtained does not contain the entire portion of the region encoding the desired restriction endonuclease, the entire encoding region can be obtained by synthesizing a primer on the basis of the base sequence of the DNA obtained, and amplifying the lacking region by PCR using the primer, or repeating screening the DNA library using the DNA fragment obtained as a probe.

It is possible to prepare a transformant containing the thus-obtained similar DNA encoding the AccIII

restriction endonuclease, to allow the transformant to express the enzyme protein encoded by the DNA, and to purify the enzyme protein expressed. Preparation of the transformant and expression and purification of the enzyme protein can be all achieved using the plasmid of the present invention. The enzyme protein thus obtained retains AccIII restriction endonuclease activity.

Additionally, the AccIII modification enzyme and DNA encoding the enzyme, both of which have not been obtained so far, can also be obtained using the above-described DNA encoding the AccIII restriction endonuclease.

For example, on the basis of the mutually close location of a restriction endonuclease gene and a modification enzyme gene in many cases, this purpose can be accomplished by obtaining a gene region encoding a protein near the restriction endonuclease gene by a DNA amplification reaction with a cassette library as a template, inserting it into an appropriate expression vector to allow the gene to be expressed, and confirming AccIII modification enzyme activity by an appropriate method. AccIII modification enzyme activity can also be confirmed, for example, on the basis of the resistance of the DNA prepared from the transformant to the cleavage activity of the AccIII restriction endonuclease. Provided that the base sequence of the above-described gene region

is determined to confirm homology to a conserved region between modification enzyme genes of a known restriction modification system [Journal of Molecular Biology, Vol. 206, pp. 305-321 (1989)], it can be anticipated to some extent before gene expression that the gene is a modification enzyme gene.

By using a cassette library of the genomic DNA of the Acc bacterium as described above, the AccIII modification enzyme and DNA encoding it can be obtained. Its amino acid sequence and base sequence are shown by SEQ ID NO:13 and SEQ ID NO:14 in the Sequence Listing, respectively.

AccIII modification enzyme herein is not limited to the above described. As stated in the description of AccIII restriction endonuclease, it is a polypeptide containing the entire or a portion of the amino acid sequence described in SEQ ID No:13 in the Sequence Listing and possessing the AccIII modification enzyme activity. Furthermore, the polypeptide resulting from at least one of deletion, addition, insertion or substitution of one or more amino acid residues in the amino acid sequence of SEQ ID NO:13 or a portion thereof in the Sequence Listing and possessing the AccIII modification enzyme activity is also included in the scope of the present invention.

A DNA encoding AccIII modification enzyme in the present invention herein comprises a DNA encoding the

amino acid sequence described in the SEQ ID NO:13 in the Sequence Listing, and includes, but not limited to, a DNA comprising the base sequence described in the SEQ ID NO:14 in the Sequence Listing, for example. Specifically, the following DNAs are within the scope of the present invention.

(1) a DNA encoding a polypeptide containing the entire or a portion of the amino acid sequence described in SEQ ID NO:13 in the Sequence Listing and possessing the AccIII modification enzyme activity;

(2) a DNA containing the entire or a portion of the DNA shown in SEQ ID NO:14 in the Sequence Listing, wherein the expression product of the DNA possesses the AccIII modification enzyme activity;

(3) a DNA encoding a polypeptide resulting from at least one of deletion, addition, insertion or substitution of one or more amino acid residues in the amino acid sequence of SEQ ID NO:13 or a portion thereof in the Sequence Listing and possessing the AccIII modification enzyme activity; and

(4) a DNA capable of hybridizing to the DNA described in above (1) to (3), and encoding a polypeptide possessing the AccIII modification enzyme activity, etc..

The present invention is hereinafter described in

more detail by means of the following reference example and working examples, which examples are not to be construed as limitative. Of the procedures described herein, basic ones regarding plasmid preparation, restriction endonuclease digestion, etc. were achieved in accordance with the methods described in Molecular Cloning: A Laboratory Manual, 2nd edition, edited by T. Maniatis et al., published by Cold Spring Harbor Laboratory, 1989.

Reference Example

(1) Culture medium and conditions

Escherichia coli was aerobically cultured at 37°C using LB medium (1% trypton, 0.5% yeast extract, 0.5% NaCl, pH 7.0). Antibiotics were each added to the medium at various concentrations depending on the plasmid retained by the *Escherichia coli* as follows: 25 μg/ml kanamycin for pFSP6, 20 μg/ml ampicillin for pXX325, 50 μg/ml ampicillin for ampicillin-resistant ColE1 type plasmid, and 100 μg/ml ampicillin for ampicillin-resistant pUC type plasmid. In the expression induction experiment, the culture broth obtained after cultivation until the stationary phase was inoculated to a fresh medium at 1%, then aerobically cultured at 37°C, after which isopropyl-β-D-thiogalactoside (IPTG) was added to a final

concentration of 0.2 mM upon reach of an OD_{600} value of 0.6 (6 x 10^8 cells/ml), followed by further cultivation.

(2) Construction of the system plasmid pFSP6

The system plasmid pFSP6 was constructed with *Escherichia coli* JM109 as a host, according to the procedure directed in Figure 8. The plasmid pSP6-2 [Nucleic Acids Research, Vol. 15, pp. 2653-2664 (1987)] was digested with HindIII (produced by Takara Shuzo) and blunted at both ends, after which it was further digested with BamHI (produced by Takara Shuzo) to yield an about 2.8 kb DNA fragment containing the SP6 RNA polymerase gene, which fragment was mixed with the plasmid vector pUC18 (produced by Takara Shuzo), previously digested with BamHI and HincII (produced by Takara Shuzo), for ligation to construct the plasmid pUCSP. Next, the plasmid pMJR1560 [Gene, Vol. 51, pp. 225-267 (1987)] was digested with KpnI and blunted at both ends, after which it was further digested with PstI (produced by Takara Shuzo) to yield an about 1.3 kb DNA fragment containing the lacIq gene, which was then isolated. The above plasmid pUCSP was digested with HindIII and blunted at both ends, after which it was further digested with PstI to yield a DNA fragment, which was mixed with the above-described about 1.3 kb DNA fragment for ligation to construct the plasmid

pUCSPlac.

Furthermore, an about 1.5 kb DNA fragment obtained by PstI digestion of pUCKm [Journal of Molecular Biology, Vol. 147, pp. 217-226 (1981)], which contains the kanamycin resistance gene, was introduced into the PstI site of the above-described plasmid pUCSPlac to construct the plasmid pUCSPlacKm. The plasmid was digested with AatII (produced by Toyobo), after which it was partially digested with NspI (produced by Takara Shuzo) to isolate an about 6.5 kb DNA fragment, which was then blunted at both ends.

On the other hand, the plasmid pXX325 [Proceedings of the National Academy of Sciences of the USA, Vol. 80, pp. 4784-4788 (1983)] was digested with HindIII and blunted at both ends, after which it was further digested with SalI to isolate an about 6.8 kb DNA fragment containing the replication origin of the miniF plasmid, which fragment was then mixed with the above-described about 6.5 kb DNA fragment for ligation to construct the plasmid pFSP6. *Escherichia coli* HB101 was transformed with the plasmid to form *Escherichia coli* HB101/pFSP6.

(3) Construction of multicopy model expression plasmids

The P_{SP6} linker, a double-stranded oligonucleotide containing the minimum region of the SP6 promoter, was

prepared from the two DNA strands whose base sequences are shown by SEQ ID NO:15 and SEQ ID NO:16 in the Sequence Listing, respectively, and was mixed with the plasmid pMS434 [Gene, Vol. 57, pp. 89-99 (1987)], previously digested with XhoI (produced by Takara Shuzo) and HindIII, for ligation to construct the model expression plasmid pMSP6L inserted the above-described promoter sequence upstream of the β-galactosidase gene on the plasmid (Figure 1).

On the other hand, by inserting a DNA fragment containing the inherent SP6 promoter sequence, obtained by digesting pSP64 [Nucleic Acids Research, Vol. 12, pp. 7035-7056 (1984)] with AccII and HindIII, into the XhoI-HindIII site of the above-described plasmid pMS434, the model expression plasmid pMSP6F was constructed (Figure 1). Furthermore, the P_{SP6}-O_{lac} linker, which contains the minimum region of the SP6 promoter and the lac operator region, was prepared from the two DNA strands whose base sequences are shown by SEQ ID NO:17 and SEQ ID NO:18 in the Sequence Listing, respectively, and was inserted between the XhoI-HindIII site of the above-described plasmid pMS434 to construct the model expression plasmid pMSP60 (Figure 2).

(4) Promoter activity of the SP6 promoter for *Escherichia*

coli RNA polymerase

The plasmids pMSP6L and pMSP6F constructed in Reference Example (3) and the plasmid pMS434 as a control, which does not contain the SP6 promoter sequence, were each introduced into the *Escherichia coli* shown in Table 1. The resulting transformants were each cultured by the method described in Reference Example (1), after which each cell culture broth was harvested during the logarithmic growth phase. The OD_{600} value of each culture broth collected was determined with a portion thereof, while the remaining portion was transferred to a pre-cooled test tube and supplemented with chloramphenicol to a final concentration of 100 mg/ml. With this culture broth as a sample, β-galactosidase activity was determined by the method described in Experiments in Molecular Genetics, edited by J.H. Miller, published by Cold Spring Harbor Laboratory, 1972.

As shown in Table 2, *Escherichia coli* MC4100 incorporating the model expression plasmid pMSP6L or pMSP6F, both harboring a sequence containing the SP6 promoter, exhibited similar level of low β-galactosidase activity, irrespective of the promoter sequence inserted. However, that incorporating the plasmid pMS434, which does not contain the SP6 promoter, exhibited only lower enzyme activity. In addition, comparing *Escherichia coli* MRi7

and MRi80 as the hosts revealed decreased β-galactosidase activity in MRi80, wherein the copy number of the plasmid was also decreased.

(5) Evaluation of the system plasmid pFSP6

Each transformant prepared in Reference Example (4) into which the system plasmid pFSP6 was further introduced, was cultured by the method described in Reference Example (1); β-galactosidase activity was determined both in a non-inductive condition and in an inductive condition before and after IPTG addition. The results were shown in Table 3. All transformants exhibited β-galactosidase activity in a non-inductive condition at a level similar to that obtained in the absence of the plasmid pFSP6 shown in Table 2.

On the other hand, with induction by IPTG addition, the β-galactosidase activity in the transformants incorporating the plasmids pMSP6L and pMSP6F was 18 to 32 times compared with that obtained in a non-inductive condition, while there was no increase in the activity in the transformants incorporating the plasmid pMS434.

(6) Effect of the lac operator sequence on expression control

Transformants obtained by introducing the plasmids

pMSP6L, pMSP6F, pMSP60 and pMS434, respectively, into *Escherichia coli* MC4100 incorporating the plasmid pFSP6 were each cultured by the method described in Reference Example (1), and β-galactosidase activity in a non-inductive condition was determined. The results are shown in Table 4. The transformant incorporating the plasmid pMSP60, which contains the lac operator sequence, still showed some β-galactosidase activity, although the activity level was lower than those obtained with the plasmids pMSP6L and pMSP6F. In short, expression in a non-inductive condition could not be completely suppressed simply by introduction of the lac operator sequence.

Table 4

Strain	β − Galactosidase Activity (Non-Inductive Condition)			
	pMSP6F	pMSP6L	pMSP60	pMS434
MC4100(pFSP6)	221	234	30	25

Example 1

(1) Culture medium and conditions

Escherichia coli was aerobically cultured at 37°C using LB medium (1% trypton, 0.5% yeast extract, 0.5% NaCl, pH 7.0). Antibiotics were each added to the medium

at various concentrations depending on the plasmid retained by the *Escherichia coli* as follows: 25 μg/ml kanamycin for pFSP6, 50 μg/ml ampicillin for ampicillin-resistant ColE1 type plasmid, 100 μg/ml ampicillin for ampicillin-resistant pUC type plasmid, 30 μg/ml chloramphenicol for chloramphenicol-resistant runaway plasmid and 30 μg/ml ampicillin for ampicillin-resistant runaway plasmid. In the expression induction experiment, the culture broth obtained after cultivation until the stationary phase was inoculated to a fresh medium at 1%, then aerobically cultured at 37°C, after which isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.2 mM upon reach of an OD_{600} value of 0.6 (6×10^8 cells/ml), followed by further cultivation.

(2) Construction of the runaway plasmid pHS2870

The runaway plasmid pHS2870, which provided a basis for construction of the expression plasmid, was constructed by the procedures shown in Figure 3. The 106 NdeI DNA fragment prepared from the two DNA strands whose base sequences are shown by SEQ ID NO:3 and SEQ ID NO:4 in the Sequence Listing, respectively, was mixed with the plasmid vector pUC19, previously digested with NdeI, for ligation. The plasmid pUC106A thus obtained was digested with PvuII and subjected to self-ligation to yield the

yield the plasmid pUC106AdPO. With this plasmid pUC106AdPO as a template, PCR was then conducted using the RNAIIA primer (whose base sequence shown by SEQ ID NO:5 in the Sequence Listing) and the 1870 primer (whose base sequence shown by SEQ ID NO:6 in the Sequence Listing) to yield an amplified DNA fragment, which was then digested with XbaI and mixed with the plasmid pSTV28, previously digested with XbaI, for ligation to yield the plasmid pHS2870. The construct of the plasmid pHS2870 is shown in Figure 4.

(3) Construction of the runaway plasmid pCRS04

After digestion with AccI (produced by Takara Shuzo) and NspI, the above-described plasmid pHS2870 was blunted at both ends and subjected to self-ligation to yield the plasmid pCRS01, which lacks the P15A replication origin. The plasmid was then digested with EcoRI (produced by Takara Shuzo) and XbaI, after which it was blunted at both ends and subjected to self-ligation in the same manner as above to construct the plasmid pCRS02. After an about 1.2 kb DNA fragment obtained by digesting the plasmid pMJR1560 with KpnI (produced by Takara Shuzo) and PstI was blunted at both ends, it was mixed with the above-described plasmid pCRS02, previously digested with NspV and VspI (both produced by Takara Shuzo) and then blunted at both

ends, for ligation to yield the plasmid pCRS04. The flow diagram of the construction of the plasmid pCRS04 is shown in Figure 5, and the construct of the plasmid pCRS04 shown in Figure 6.

(4) Construction of the runaway expression vector pACE601

The above-described plasmid pCRS04, previously digested with NheI (produced by Takara Shuzo) and subsequently blunted at both ends, was mixed with the P_{SP6}-O_{lac} EX linker, prepared from the two DNA strands whose base sequences are shown by SEQ ID NO:7 and SEQ ID NO:8 in the Sequence Listing, for ligation to construct the plasmid pACE601. The flow diagram of the construction of the plasmid pACE601 is shown in Figure 9. The construction of these plasmids were conducted with *Escherichia coli* JM109 as a host.

(5) Construction of the runaway expression vector pACE611

The runaway expression vector pACE601 was digested with XhoI-HindIII, and the P_{SP6} linker, consisting of the synthetic oligo-DNAs shown by SEQ ID NO:15 and SEQ ID NO:16 in the Sequence Listing, was inserted into that site to construct the runaway expression vector pACE611. The construct of pACE611 is shown in Figure 10. The flow diagram of the construction of the plasmid pACE611 is

shown in Figure 11.

(6) Construction of the runaway expression vectors pACE701 and pACE702

An about 1 kb DNA fragment obtained by digesting the plasmid vector pUC118 (produced by Takara Shuzo) with BspHI (produced by NEB) was blunted at both ends, after which it was mixed with the above-described plasmid pCRS04, previously digested with NheI, and blunted at both ends, for ligation to construct the plasmid pCRS70. The flow diagram of the construction of the plasmid pCRS70 is shown in Figure 12. Next, the plasmid was digested with NcoI (produced by Takara Shuzo) and BsaAI (produced by NEB), after which it was blunted at both ends and mixed with the above-described P_{SP6}-O_{lac} EX linker for ligation to construct two plasmids pACE701 and pACE702, which incorporate the linker inserted in mutually opposite directions. The flow diagrams of the construction of the plasmids pACE701 and pACE702 are shown in Figure 13. The construction of these plasmids were conducted with *Escherichia coli* JM109 as a host.

(7) Construction of model runaway expression plasmids

PCR was conducted using the primers trpA-N-NcoI and lacZ-C-NcoI with the above-described plasmid pMS434 as a

template, to yield a DNA fragment containing the β-galactosidase gene. The base sequences of the primers trpA-N-NcoI and lacZ-C-NcoI are shown by SEQ ID NO:9 and SEQ ID NO:10 in the Sequence Listing, respectively. After digestion with NcoI, the fragment was mixed with each of the above-described plasmids pACE601, pACE701 and pACE702, all previously digested with NcoI, for ligation to yield the model runaway expression plasmids pACE601Z, pACE701Z and pACE702Z, all incorporating the β-galactosidase gene introduced downstream of the P_{SP6}-O_{lac} sequence. The construction of these plasmids were conducted with *Escherichia coli* JM109 as a host.

(8) Evaluation of the expression levels of runaway expression plasmid in a non-inductive condition

The transformants MC4100/pFSP6/pACE601Z, MC4100/pFSP6/pACE701Z and MC4100/pFSP6/pACE702Z, which were obtained after introducing the above-described model runaway expression plasmids pACE601Z, pACE701Z and pACE702Z, respectively into *Escherichia coli* MC4100 incorporating the above-described system plasmid pFSP6 (hereinafter referred to as MC4100/PFSP6), were each cultured under the conditions described in Example (1); each culture broth collected during the logarithmic growth phase was assayed for β-galactosidase activity by the

method described in Reference Example (4). In all transformants examined, β-galactosidase activity was below the detection limit, demonstrating a greater expression-suppressing effect than that obtained with the multicopy plasmids shown in Reference Example (4).

(9) Construction of the Nsp7524 III restriction endonuclease gene expression plasmid

The plasmid pBRN3, which contains the Nsp7524 III restriction modification system gene, was prepared from *Escherichia coli* MC1061/pBRN3 (FERM BP-5741). PCR was conducted using the primers L-ORF and NspR-ORF3 with this plasmid as a template to yield an about 1 kb DNA fragment containing the Nsp7524 III restriction endonuclease gene alone. The base sequences of the primers L-ORF and NspR-ORF3 are shown by SEQ ID NO:11 and SEQ ID NO:12 in the Sequence Listing, respectively. After digestion with NcoI, the fragment was mixed with the above-described plasmid pACE601, previously digested with NcoI, for ligation to construct the plasmid pACE601-NspIII, which incorporates the Nsp7524 III restriction enzyme gene alone introduced downstream of the P_{SP6}-O_{lac} sequence. The plasmid was stably retained in *Escherichia coli* JM109 not containing the Nsp7524 III modification enzyme gene.

(10) Construction of Nsp7524 III restriction endonuclease gene expression system

The transformants HB101/pFSP6/pACE601-NspIII and HB101/pFSP6/pACE601, which were obtained after introducing the above-described plasmid pACE601-NspIII, which contains the Nsp7524 III restriction endonuclease gene, and the control plasmid pACE601 into *Escherichia coli* HB101 incorporating the above-described system plasmid pFSP6 (hereinafter referred to as HB101/PFSP6), were each cultured in LB medium until the stationary phase; each culture broth collected was inoculated to two tubes of fresh medium at 1% and aerobically cultured at 37°C. Upon reach of an OD_{600} value of 0.6, IPTG was added to one of the tubes to a final concentration of 0.2 mM, followed by further cultivation. After completion of the cultivation, cells were harvested, suspended in cell disruption buffer A (20 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol), and disrupted by ultrasonication, followed by centrifugation to yield a crude extract.

A 1 µl portion of this crude extract was added to 30 µl of a reaction mixture (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl, 1 µg λ-DNA) and reacted at 37°C for 1 hour, after which the reaction mixture was subjected to agarose gel electrophoresis to examine for the digestion of λ-DNA and confirm restriction

endonuclease activity. As shown in Figure 14, restriction endonuclease activity was observed only in HB101/pFSP6/pACE601-NspIII with expression induced by the addition of IPTG, and its λ-DNA cleavage pattern agreed with that of AvaI, an isoschizomer of Nsp7524 III.

The results of the above-described λ-DNA digestion reaction as conducted for extended periods of 2 and 3 hours are shown in Figure 15. In this case, because the crude extract used in the present experiment contained nuclease derived from the host *Escherichia coli*, the DNA fragment resulting from Nsp7524 III activity underwent further degradation by *Escherichia coli* nuclease, resulting in bands whose density decreased as the increase in reaction time, on lanes 8 through 10, with no bands detected on lane 10. On lanes 11 through 13, the Nsp7524 III digestion fragment was not produced because of the absence of Nsp7524 III induction even when reaction time was extended, demonstrating that the λ-DNA was made into the lower molecular weight fragments by the direct action of *Escherichia coli* nuclease. No restriction endonuclease activity was detected in the crude extract from HB101/pFSP6/pACEA601.

Example 2

Isolation and expression of the AccIII restriction

endonuclease gene

(1) Determination of N-terminal amino acid sequence of AccIII restriction endonuclease protein and synthesis of primer DNA corresponding to the amino acid sequence

About 2 ku of a commercial product of the AccIII restriction endonuclease (produced by Takara Shuzo) was subjected to gel filtration using a column of Sephacryl S300 (produced by Pharmacia) to determine the molecular weight of the AccIII restriction endonuclease. Judging from the elution position where activity was detected, the molecular weight of the AccIII restriction endonuclease was proven to be about 70,000. Because most restriction endonuclease proteins are dimers, the AccIII restriction endonuclease protein was expected to be mobilized to a position for a molecular weight of about 35,000 in SDS polyacrylamide gel electrophoresis.

Next, to obtain a sample for N-terminal amino acid sequencing of the AccIII restriction endonuclease protein, about 2 ku of a commercial product of the AccIII restriction endonuclease, together with a protein molecular weight marker, was subjected to SDS polyacrylamide gel electrophoresis, after which the protein was transferred from the gel to a PVDF membrane and stained with bromophenol blue to confirm the protein

position. After destaining with 10% acetic acid-50% methanol, the portion of the PVDF membrane where a protein of about 35,000 molecular weight was blotted was cut out and subjected to automatic Edman degradation using the protein sequencer G1000A (produced by Hewlett-Packard) to determine the N-terminal amino acid sequence shown by SEQ ID NO:19 in the Sequence Listing. On the basis of this sequence, AccIII primers 1 and 2, shown by SEQ ID NO:20 and SEQ ID NO:21 in the Sequence Listing, respectively, were then synthesized for use as a pair of cassette primers.

(2) Preparation of genomic DNA of Acc bacterium

In accordance with the method of Kita et al., described in Nucleic Acids Research, Vol. 13, pp. 8685-8694 (1985), the Acc bacterium was cultured to obtain wet cells. Two grams of the wet cells obtained was suspended in 10 ml of buffer B [25 mM Tris-HCl (pH 8.0), 50 mM glucose, 10 mM EDTA], stirred in the presence of 1 ml of a lysozyme solution prepared to 2 mg/ml in buffer B, and kept standing at 37°C for 20 minutes. Next, 28 ml of buffer C [100 mM NaCl, 100 mM Tris-HCl (pH 8.0)] was added to this solution, followed by stirring. One milliliter of a proteinase K solution prepared to 20 mg/ml in TE [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)] and 4 ml of a 10% SDS

solution were further added, followed by stirring, after which the mixed solution was kept standing at 37°C for 1 hour.

To this solution, 6 ml of a 5 M aqueous solution of NaCl and 6 ml of buffer D [10% CTAB (cetyl trimethyl ammonium bromide), 0.7 M NaCl] was added, followed by stirring, after which the mixed solution was kept standing at 60°C for 20 minutes. This solution was treated with phenol and subsequently with chloroform, after which the water layer was separated. To the water layer, 50 µl of an RNaseA (produced by Sigma) solution prepared to 10 mg/ml in TE was added, followed by stirring, after which the mixed solution was kept standing at 37°C for 40 minutes. After being kept standing, this solution was treated with phenol and subsequently with chloroform, after which the water layer was separated. To the water layer, an equal volume of cold ethanol was added; the DNA precipitated was recovered by winding around a glass capillary. The DNA was washed with 70% ethanol and dissolved in 3 ml of TE to yield about 200 µg of genomic DNA.

(3) Preparation of Acc genomic cassette library

The following procedures were conducted basically in accordance with the method described on pages F16-F17 in

"Gene Engineering Guide" (1995-1996 edition), Takara Shuzo.

The genomic DNA obtained in (2) was completely digested with EcoRI (produced by Takara Shuzo); the DNA fragment obtained was ligated with an EcoRI cassette (produced by Takara Shuzo), having a cohesive end complementary thereto, to yield an EcoRI cassette library. Similarly, the genomic DNA was completely digested separately with the restriction endonucleases BglII, EcoT14I, EcoT22I, HindIII, PstI, SalI and XbaI (all produced by Takara Shuzo). The genomic DNA fragments obtained were each bound to each of several cassettes (produced by Takara Shuzo), having a complementary protruding end, to yield the BglII, EcoT14I, EcoT22I, HindIII, PstI, SalI and XbaI cassette libraries, respectively. These cassette libraries are generically referred to as the Acc genomic cassette library.

(4) Analysis of the AccIII restriction endonuclease gene

A PCR-based DNA amplification reaction was carried out using a primer pair of AccIII primer 1 and cassette primer C1 (produced by Takara Shuzo) with the EcoRI cassette library obtained in (3) as a template. To efficiently and specifically amplify the desired region, a second PCR-based DNA amplification reaction was carried

out using a primer pair of AccIII primer 2 and cassette primer C2 (produced by Takara Shuzo) with a portion of the reaction liquid obtained, to yield an amplified DNA fragment. Similarly, with the BglII, EcoT14I, EcoT22I, HindIII, PstI, SalI and XbaI cassette libraries as a template respectively, the above two-step PCR-based DNA amplification reaction was carried out separatedly to yield amplified DNA fragments. Each amplified DNA was analyzed by agarose gel electrophoresis; the amplified DNA fragment derived from the XbaI cassette library showed a particularly high amplification efficiency.

With this in mind, a PCR-based DNA amplification reaction was again carried out using a primer pair of AccIII primer 1 and cassette primer C1 with the XbaI cassette library as a template. This reaction mixture was subjected to agarose gel electrophoresis; an about 0.5 kb amplified DNA fragment was recovered from the gel. A second PCR-based DNA amplification reaction was carried out using a primer pair of AccIII primer 2 and cassette primer C2 with the DNA fragment obtained as a template. Base sequencing of the about 0.5 kb amplified DNA fragment obtained demonstrated that the fragment encodes a portion of the protein whose N-terminal amino acid sequence was determined in (1). On the other hand, the putative molecular weight of the AccIII restriction endonuclease

protein is about 35,000, and the gene encoding the protein is assumed to be about 1 kb in length. It was therefore expected that the base sequence of the AccIII restriction endonuclease gene could be determined, provided that information on the accurate base sequence of the 5'-terminal region to which AccIII primers 1 and 2 annealed, and information on the remaining about 0.5 kb base sequence in the 3' region, were available, in addition to the above-described information on the about 0.5 kb base sequence.

With this in mind, to determine the accurate base sequence of the 5'-terminal region of the AccIII restriction endonuclease gene region, AccIII primer 3, shown by SEQ ID NO:22 in the Sequence Listing, and AccIII primer 4, shown by SEQ ID NO:23 in the Sequence Listing, were synthesized. Next, a PCR-based DNA amplification reaction was carried out using a primer pair of AccIII primer 4 and cassette primer C1 with the EcoRI cassette library obtained in (3) as a template. A PCR-based DNA amplification reaction was carried out using a primer pair of AccIII primer 3 and cassette primer C2 with a portion of this reaction mixture. Similarly, with the BglII, EcoT14I, EcoT22I, HindIII, PstI, SalI and XbaI cassette libraries as a template respectively, the above two-step PCR-based DNA amplification reaction was separatedly

carried out. Of the DNA fragments amplified, the shortest, i.e., the about 1 kb DNA fragment was obtained with the EcoT14I cassette library as a template, the base sequence thereof was determined.

Furthermore, to determine the about 0.5 kb sequence on the 3' side of the gene region assumed to encode the AccIII restriction endonuclease protein, AccIII primer 5, shown by SEQ ID NO:24 in the Sequence Listing, and AccIII primer 6, shown by SEQ ID NO:25 in the Sequence Listing, were synthesized. A PCR-based DNA amplification reaction was carried out using a primer pair of AccIII primer 5 and cassette primer C1 with the EcoRI cassette library obtained in (3) as a template. A PCR-based DNA amplification reaction was carried out using a primer pair of AccIII primer 6 and cassette primer C2 with a portion of this reaction mixture. Similarly, with the BglII, EcoT14I, EcoT22I, HindIII, PstI, SalI and XbaI cassette libraries as a template respectively, the above two-step PCR-based DNA amplification reaction was carried out separatedly.

Of the DNA fragments amplified, the about 0.5 kb and about 0.8 kb DNA fragments each obtained with the EcoT22I and HindIII cassette libraries as a template respectively were subjected to a base sequencing. Combining the results for the three base sequences determined, the base

sequence information on an about 1.6 kb DNA fragment was obtained. Its base sequence is shown by SEQ ID NO:26 in the Sequence Listing. Furthermore, searching for an open reading frame (ORF) capable of encoding the protein demonstrated the presence of ORF1 at base numbers 558 through 1442, a portion thought to be ORF2 at base numbers 1588 through 1434, and a portion thought to be ORF3 at base numbers 1 through 535. Encoding the protein whose N-terminal amino acid sequence was determined in (1), ORF1 was deemed to be the AccIII restriction endonuclease gene. The base sequence of the AccIII restriction endonuclease gene is shown by SEQ ID NO:2 in the Sequence Listing, wherein the fourth base, as counted from the 5' terminus, is C. The amino acid sequence deduced from the base sequence is shown by SEQ ID NO:1 in the Sequence Listing, wherein the second amino acid, as counted from the N terminus, is Leu. The direction of translation of ORF2 is opposite that of ORF1 and ORF3.

(5) Construction of the plasmid pCRA19

Next, a plasmid for expression of the AccIII restriction endonuclease gene was constructed. First, to obtain the gene, the primer Acc-RL, shown by SEQ ID NO:27 in the Sequence Listing, and the primer Acc-RR, shown by SEQ ID NO:28 in the Sequence Listing, were synthesized. A

restriction endonuclease NcoI recognition sequence site was introduced into both primers. Using the above described primer pair, it is possible to cut out the AccIII restriction endonuclease gene from an amplified DNA fragment obtained by PCR with the genomic DNA of the Acc bacterium as a template, using the restriction endonuclease NcoI, and the translation codon frames coincide with each other under control of the SP6 promoter when the gene is inserted into the NcoI site of the pACE611 vector. On the other hand, a use of this primer pair results in replacement of the fourth base, as counted from the 5' terminus of the AccIII restriction endonuclease gene, from C to G, and of the second amino acid, as counted from the N terminus of the protein encoded by the gene, from Leu to Val. Using the above primer pair with the genomic DNA of the Acc bacterium obtained in (2) as a template, a PCR-based DNA amplification reaction was carried out. This DNA fragment was completely digested with the restriction endonuclease NcoI (produced by Takara Shuzo), after which it was subjected to agarose gel electrophoresis; a DNA fragment of about 900 bp size was recovered. Next, this DNA fragment was inserted into the NcoI site of the pACE611 vector so that it was located downstream of the SP6 promoter.

This recombinant DNA was introduced into *Escherichia coli* JM109 to yield transformants. Thirty transformants were randomly selected, and each inoculated to 5 ml of an LB medium containing 30 μg/ml chloramphenicol and cultured at 37°C. When the OD_{600} value of the cell culture broth reached 0.6, IPTG was added to a final concentration of 2 mM to increase the plasmid copy number, followed by further cultivation at 37°C for 2 hours, after which plasmid DNA was prepared from each culture. Each plasmid DNA obtained was simultaneously cleaved with the restriction endonucleases HindIII and XbaI, followed by confirmation of the length of the resulting DNA fragments by agarose gel electrophoresis; a plasmid thought to contain the gene region inserted in the right direction was detected. This plasmid, designated pCRA19, was introduced into *Escherichia coli* JM109 to yield a transformant, which was designated *Escherichia coli* JM109/pCRA19.

(6) Construction of expression system for the AccIII restriction endonuclease gene

First, the SP6 RNA polymerase gene was introduced into *Escherichia coli* JM109 to construct a phage vector allowing further expression of the gene. Specifically, by inserting an SP6 RNA polymerase gene fragment obtained by

BamHI-HindIII digestion of pSP6-2 into the BamHI-HindIII site within the multicloning site of a commercial product of the phage vector M13mp18 (produced by Takara Shuzo), the SP6 RNA polymerase expression phage M13sp6 was constructed.

Next, the transformant *Escherichia coli* JM109/pCRA19 was inoculated to 5 ml of an LB medium containing 30 μg/ml chloramphenicol and cultured at 37°C. When the OD_{600} value of the cell culture broth reached 0.6, IPTG was added to a final concentration of 2 mM to increase the plasmid copy number, followed by further cultivation at 37°C for 2 hours. These cells were then infected with the phage M13sp6 to express the protein encoded by the AccIII restriction endonuclease gene, followed by further cultivation at 37°C for 16 hours. A 11 mg portion of the wet cells obtained was suspended in 180 μl of cell disruption buffer E [20 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol], after which the cells were disrupted by ultrasonication, followed by centrifugation (18000 g, 10 minutes) to separate the solid and liquid.

Determining the activity of the supernatant under the activity determination conditions shown on the data sheet attached to the AccIII restriction endonuclease produced by Takara Shuzo demonstrated the production of the AccIII restriction endonuclease in an amount of about 8000 units

per gram of wet cells, a level about 16 times that obtained with the Acc bacterium, per unit weight of wet cells. Neither activity of restriction endonucleases other than AccIII nor AccIII modification enzyme activity was noted in the supernatant obtained. Regarding the AccIII restriction endonuclease gene inserted into the plasmid pCRA19, it was demonstrated that the fourth base, as counted from the translation initiation base, was replaced from C to G, upon DNA amplification by PCR, resulting in the replacement of the second amino acid, as counted from the N-terminus of the translated protein, from Leu to Val, and that the protein possesses AccIII restriction endonuclease activity.

An isolation/mass production system for the AccIII restriction endonuclease gene in the absence of AccIII modification enzyme was thus developed.

(7) Isolation of the AccIII modification enzyme gene

Modification enzyme genes and restriction endonuclease genes are often closely located. With this in mind, to determine the ORF2 region deduced in (4) above, the base sequence of an about 1.4 kb DNA fragment obtained using the EcoRI cassette library as a template, out of the amplified DNA fragments prepared to determine the 3' region of the AccIII restriction endonuclease gene

in (4) above, was determined. Next, to determine the ORF3 region deduced in (4) above, the base sequence of an about 2.2 kb DNA fragment obtained using the BglII cassette library as a template, out of the amplified DNA fragments prepared to determine the 5'-terminal region of the AccIII restriction endonuclease gene in (4) above, was determined. Combining these base sequences and the base sequence of the about 1.6 kb DNA fragment containing the AccIII restriction endonuclease gene region determined in (4) resulted in the information on the about 4.2 kb base sequence shown by SEQ ID NO:29 in the Sequence Listing. The AccIII restriction endonuclease gene was located at base numbers 1913 through 2797, ORF2 at base numbers 3712 through 2789, and ORF3 at base numbers 691 through 1890. Of these ORFs, ORF2 proved to contain a portion highly homologous to the conserved region among modification enzymes in a known restriction modification system.

Next, to obtain the ORF2 region, an about 1.1 kb ORF2-containing portion was cut out using EcoT22I and HpaI (produced by Takara Shuzo) from an amplified DNA fragment obtained by two-step PCR using a primer pair of Acc primer 6 and cassette primer C1 and another primer pair of Acc primer 5 and cassette primer C2 in the respective steps, with the EcoRI cassette library as a template, and was inserted into the SmaI site downstream of the lac promoter

in the pUC118 vector. If this recombinant plasmid contains the AccIII modification enzyme gene, and if the AccIII modification enzyme can be expressed in *Escherichia coli*, the DNA in the culture of the transformant obtained by introducing this recombinant plasmid into *Escherichia coli* JM109 would undergo methylation by the AccIII modification enzyme and acquire resistance to cleavage by the AccIII restriction endonuclease.

Because the pUC118 vector used to construct this recombinant plasmid has no AccIII restriction endonuclease recognition sequence, however, it is inappropriate to use this recombinant plasmid by itself to confirm AccIII modification enzyme activity. On the other hand, there is an AccIII restriction endonuclease recognition sequence in the plasmid pSTV29, which can be co-present with this recombinant plasmid in *Escherichia coli* JM109. With this in mind, to utilize pSTV29 as an index of expression of the AccIII modification enzyme, the above recombinant plasmid and pSTV29 were both introduced into *Escherichia coli* JM109 to yield transformants. Three transformants were each cultured at 37°C for 16 hours in 2 ml of an LB medium containing 100 μg/ml ampicillin, 30 μg/ml chloramphenicol and 2 mM IPTG, after which plasmid DNA was prepared from each culture.

The DNA thus prepared is available as a mixture of

pSTV29 and the above-described recombinant plasmid. When each DNA sample was subjected to a digestion with the AccIII restriction endonuclease, the pSTV29 in all samples exhibited resistance to the AccIII restriction endonuclease activity, demonstrating the insertion of the AccIII modification enzyme gene into the recombinant plasmid contained in the DNA sample. Furthermore, when the DNA sample was simultaneously cleaved with the restriction endonucleases HindIII and XbaI, followed by analysis of the length of the resulting DNA fragments by agarose gel electrophoresis, the presence of ORF2 in the recombinant plasmid was confirmed. ORF2 was thus proven to be the AccIII modification enzyme gene. The base sequence of the AccIII modification enzyme gene obtained and the amino acid sequence deduced therefrom are shown by SEQ ID NO:14 and SEQ ID NO:13 in the Sequence Listing, respectively.

The structure of the AccIII restriction modification system gene demonstrated according to the present invention is shown in Figure 16, wherein M, R and the arrow represent the modification enzyme gene, the restriction endonuclease gene, and the orientation of ORF, respectively.

INDUSTRIAL APPLICABILITY

The present invention provides a plasmid vector capable of introducing into a host an exogenous desired gene encoding a protein lethal or harmful to the host, and a method for being capable of efficiently expressing the protein using the plasmid vector for the first time. A method for being capable of isolating a restriction endonuclease gene which constitutes a restriction modification system without co-existence of a modification enzyme gene, which has been difficult in the prior arts, is also provided. Furthermore, an AccIII restriction endonuclease gene and an AccIII modification enzyme gene are isolated by the present invention, and, from *Escherichia coli* transformed with the plasmid containing the gene, it is possible to easily obtain an AccIII restriction endonuclease or an AccIII modification enzyme available in the genetic engineering at a desired purity of an enzyme preparation compared to the prior method for purification.

SEQUENCE LISTING

SEQ ID NO:1
SEQUENCE LENGTH: 295
SEQUENCE TYPE: amino acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: peptide
FEATURE: 2 (Val or Leu)
SEQUENCE DESCRIPTION:

```
Met Xaa Pro Leu Asp Lys Asp Leu Gln Lys Ala Lys Ile Ser Ile
  1               5                  10                  15
Thr Asp Phe Phe Glu Ile Thr Asn Arg Val Leu Asp Tyr Phe Pro
                20                  25                  30
Asn Val Ile Asn Asn Thr Val Glu Lys Gly Asp Tyr Leu Ile Ser
                35                  40                  45
Ser Ser Asn Ile Ala Gly Thr Ile Lys Phe Leu Arg Pro Ile Asn
                50                  55                  60
Arg Lys Leu Phe Ile Gln Glu Lys Lys Val Phe Asn Asp Tyr Phe
                65                  70                  75
Gln Lys Leu Ile Ile Val Phe Glu Asn Ile Arg Asn Lys Lys Thr
                80                  85                  90
Val Thr Glu Glu Asp Lys Ile Ile Ile Asp Arg Val Ile Tyr Thr
                95                 100                 105
Ile Gln Gln Ser Ile Gly Ile Gly Leu Asp Leu Met Val Asn Gln
               110                 115                 120
Asn Ser Ala Arg Lys His Val Gly Asn Arg Phe Glu Glu Leu Ile
               125                 130                 135
Arg Val Ile Phe Thr Glu Ile Ser Val Ser Asn Lys Arg Thr Val
               140                 145                 150
Leu Gln Ile Pro Tyr Glu Thr Asp Glu Gly Gln Lys Ile Tyr Lys
               155                 160                 165
Cys Glu Asn Asp Leu Ile Ile Ser Pro Phe Glu Asn Val Glu Ser
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                170                 175                 180
Thr Asn Lys His Leu Asp Glu Asn Glu Ile Val Val Ser Ile Lys
                185                 190                 195
Thr Thr Ser Lys Asp Arg Met Gly Lys Met Phe Ile Asp Lys Ile
                200                 205                 210
Leu Leu Glu Arg Phe Val Lys His Pro Gln Lys Val Ile Gly Ile
                215                 220                 225
Phe Leu Asn Asp Val Gln Arg Lys Glu Asp Asn Asn Ile Ser Phe
                230                 235                 240
Thr Leu Val Ser Gly Leu Phe Met Val Tyr Thr Lys Phe Leu Thr
                245                 250                 255
Thr Leu Glu Gly Ile Tyr Tyr Leu Asp Pro Pro Pro Asn Ala Leu
                260                 265                 270
Lys Leu Pro Tyr Ser Asn His Met Lys Arg Phe Ser Asp Leu Ile
                275                 280                 285
Thr Glu Asp Leu Glu Lys Leu Phe Ser Ser
                290                 295
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SEQ ID NO:2
SEQUENCE LENGTH: 885
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: Genomic DNA
SEQUENCE DESCRIPTION:

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ATGSTACCAC TGGATAAAGA TTTACAAAAA GCAAAGATTT CAATTACTGA TTTTTTTGAA   60
ATTACAAATA GAGTTTTAGA TTATTTCCCC AATGTAATCA ATAATACAGT TGAAAAAGGA  120
GATTATTTAA TATCCTCATC AAATATTGCT GGAACAATAA AATTCCTAAG ACCAATCAAT  180
AGAAAGTTAT TTATTCAGGA AAAAAAAGTT TTCAATGATT ATTTTCAAAA ACTGATTATA  240
GTTTTTGAAA ATATAAGGAA CAAAAAAACT GTAACAGAGG AAGATAAAAT TATTATTGAT  300
AGGGTAATTT ACACAATACA GCAATCTATT GGAATTGGTT TAGATTTAAT GGTTAATCAA  360
AATAGTGCTA GAAAGCACGT TGGTAACCGA TTTGAAGAAT TAATTAGAGT CATTTTTACA  420
GAAATATCAG TATCGAATAA AAGAACTGTA TTACAAATTC CATATGAAAC TGATGAAGGA  480
CAGAAAATTT ACAAATGCGA GAATGACCTC ATTATTTCTC CTTTTGAAAA TGTAGAATCT  540
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ACAAACAAAC ATCTAGATGA AAATGAGATT GTTGTTTCAA TAAAGACAAC ATCAAAAGAT 600
AGGATGGGAA AAATGTTTAT AGATAAAATT TTACTTGAAA GGTTTGTTAA ACACCCTCAA 660
AAAGTTATAG GGATTTTCCT CAATGATGTA CAAAGAAAAG AAGACAACAA TATCAGCTTT 720
ACACTTGTTT CAGGATTATT TATGGTGTAT ACTAAATTCT TAACTACTCT TGAAGGGATC 780
TATTATTTAG ATCCACCACC TAATGCATTG AAACTACCAT ATTCTAATCA TATGAAAAGA 840
TTTTCAGATT TAATTACAGA AGACCTTGAA AAATTATTCT CCTCT 885

SEQ ID NO:3
SEQUENCE LENGTH: 215
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
TATGGATATG TTCATAAACA CGCATGTAGG CAGATAGATC TTTGGTTGTG AATCGCAACC 60
AGTGGCCTTA TGGCAGGAGC CGCGGATCAC CTACCATCCC TAATGACCTG CAGGCATGCA 120
AGCTTGCATG CCTGCAGGTC ATTAGGTACG GCAGGTGTGC TCGAGGCGAA GGAGTGCCTG 180
CATGCGTTTC TCCTTGGCTT TTTTCCTCTG GGACA 215

SEQ ID NO:4
SEQUENCE LENGTH: 215
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
TATGTCCCAG AGGAAAAAAG CCAAGGAGAA ACGCATGCAG GCACTCCTTC GCCTCGAGCA 60
CACCTGCCGT ACCTAATGAC CTGCAGGCAT GCAAGCTTGC ATGCCTGCAG GTCATTAGGG 120
ATGGTAGGTG ATCCGCGGCT CCTGCCATAA GGCCACTGGT TGCGATTCAC AACCAAAGAT 180
CTATCTGCCT ACATGCGTGT TTATGAACAT ATCCA 215

SEQ ID NO:5
SEQUENCE LENGTH: 28

SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
AGATCTAGAG CAAACAAAAA AACCACCG 28

SEQ ID NO:6
SEQUENCE LENGTH: 24
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
GGTCTAGATC CCAGAGGAAA AAAG 24

SEQ ID NO:7
SEQUENCE LENGTH: 100
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
CTCGAGATTT AGGTGACACT ATAGAATACG GAATTGTGAG CGGATAACAA TTCCAAGCTT 60
CACAGGAAAC AGACCATGGC TTAAGTAACT AGTGAATTCG 100

SEQ ID NO:8
SEQUENCE LENGTH: 100
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
CGAATTCACT AGTTACTTAA GCCATGGTCT GTTTCCTGTG AAGCTTGGAA TTGTTATCCG 60

CTCACAATTC CGTATTCTAT AGTGTCACCT AAATCTCGAG 100

SEQ ID NO:9
SEQUENCE LENGTH: 27
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
AATCCCATGG AACGCTACGA ATCTCTG 27

SEQ ID NO:10
SEQUENCE LENGTH: 29
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
CCGGCCATGG TTATTTTTGA CACCAGACC 29

SEQ ID NO:11
SEQUENCE LENGTH: 26
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
TAACTTGAAT CCATGGGTTC TCACCG 26

SEQ ID NO:12
SEQUENCE LENGTH: 29
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
TACTCAGTAG CCATGGCTCT CATAGACCG 29

SEQ ID NO:13
SEQUENCE LENGTH: 308
SEQUENCE TYPE: amino acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: peptide
SEQUENCE DESCRIPTION:

```
Met Asn Glu Ile Ala Phe Asp Asn Tyr Ser Tyr Ile Pro Lys Leu
  1               5                  10                  15
Lys Leu Tyr Ser Glu Ile Glu Leu Lys Pro Phe Phe Ile Ser Lys
                 20                  25                  30
Asn Gly Ser Leu Phe Asn Val Asp Ala Ile Asp Phe Leu Arg Lys
                 35                  40                  45
Leu Glu Ser Asn Ser Val Asp Leu Ile Phe Ala Asp Pro Pro Tyr
                 50                  55                  60
Asn Ile Lys Lys Ala Glu Trp Asp Ile Phe Ser Ser Gln Asn Glu
                 65                  70                  75
Tyr Leu Glu Trp Ser Lys Glu Trp Ile Met Glu Ala His Arg Val
                 80                  85                  90
Leu Lys Asp Asn Gly Ser Leu Tyr Val Cys Gly Phe Ser Glu Ile
                 95                 100                 105
Leu Ala Asp Ile Lys Phe Ile Thr Ser Lys Tyr Phe His Ser Cys
                110                 115                 120
Lys Trp Leu Ile Trp Phe Tyr Arg Asn Lys Ala Asn Leu Gly Lys
                125                 130                 135
Asp Trp Gly Arg Ser His Glu Ser Ile Leu Leu Leu Arg Lys Ser
                140                 145                 150
Lys Asn Phe Ile Phe Asn Ile Asp Glu Ala Arg Ile Pro Tyr Asn
                155                 160                 165
Glu His Thr Val Lys Tyr Pro Gln Arg Thr Gln Ala Glu Ser Ser
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                  170                 175                 180
Gln Tyr Ser Asn Ser Lys Lys Gln Tyr Ile Trp Glu Pro Asn Pro
                  185                 190                 195
Leu Gly Ala Lys Pro Lys Asp Val Leu Glu Ile Pro Thr Ile Ser
                  200                 205                 210
Asn Gly Ser Trp Glu Arg Ser Ile His Pro Thr Gln Lys Pro Val
                  215                 220                 225
Glu Leu Leu Lys Lys Ile Ile Leu Ser Ser Ser Asn Lys Asp Ser
                  230                 235                 240
Leu Ile Leu Asp Pro Phe Gly Gly Ser Gly Thr Thr Tyr Ala Val
                  245                 250                 255
Ala Glu Ala Phe Gly Arg Lys Trp Ile Gly Thr Glu Leu Asp Lys
                  260                 265                 270
Asn Tyr Cys Leu Glu Ile Gln Lys Arg Leu Lys Asp Glu Ser Met
                  275                 280                 285
Ile Asn Arg Ile Phe Ser Gly Asp Asp Asp Ser Asn Ser Gln Asn
                  290                 295                 300
Arg Arg Lys Lys Leu Arg Gly Glu
                  305
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SEQ ID NO:14
SEQUENCE LENGTH: 924
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: Genomic DNA
SEQUENCE DESCRIPTION:

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GTGAATGAAA TAGCGTTTGA TAATTACAGT TATATACCAA AATTAAAACT TTATTCGGAA   60
ATCGAGCTTA AACCATTTTT TATTTCAAAA AACGGTTCAC TTTTCAATGT TGATGCTATT  120
GATTTTTTAA GAAAATTAGA GAGTAATTCT GTGGATTTAA TTTTTGCAGA TCCACCTTAT  180
AACATTAAAA AGGCAGAGTG GGATATTTTT TCTTCTCAAA ATGAATATCT CGAATGGAGT  240
AAAGAATGGA TAATGGAAGC TCATAGAGTT TTAAAAGATA ATGGCAGTTT ATATGTTTGT  300
GGCTTTTCAG AAATTCTGGC AGACATAAAA TTTATCACTT CAAAATATTT TCACAGTTGT  360
AAATGGTTGA TTTGGTTCTA TAGAAACAAG GCAAATTTAG GTAAAGATTG GGGACGTTCA  420
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CACGAAAGTA TACTGTTATT AAGAAAATCT AAAAATTTTA TTTTTAATAT TGATGAGGCA 480
CGAATCCCGT ATAATGAGCA TACAGTTAAA TATCCACAAA GAACCCAGGC CGAATCTTCG 540
CAATATTCGA ACTCAAAAAA GCAATATATT TGGGAGCCAA ACCCATTAGG AGCTAAGCCA 600
AAAGATGTTT TGGAGATTCC CACAATTTCA AATGGTTCTT GGGAAAGAAG TATTCACCCT 660
ACGCAAAAGC CAGTAGAATT GCTTAAAAAA ATAATTTTAT CTTCATCTAA TAAAGATAGT 720
TTAATTCTTG ATCCATTTGG TGGTTCGGGA ACTACATATG CTGTTGCGGA AGCTTTTGGC 780
AGAAAATGGA TTGGAACAGA GTTAGATAAA AATTATTGTC TGGAAATTCA AAAGCGATTG 840
AAAGACGAAA GTATGATCAA CAGGATTTTT TCAGGCGATG ATGATTCAAA TTCTCAAAAT 900
AGAAGAAAAA AATTAAGAGG AGAA 924

SEQ ID NO:15
SEQUENCE LENGTH: 29
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
TCGAGATTTA GGTGACACTA TAGAATACA 29

SEQ ID NO:16
SEQUENCE LENGTH: 29
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
AGCTTGTATT CTATAGTGTC ACCTAAATC 29

SEQ ID NO:17
SEQUENCE LENGTH: 54
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:
TCGAGATTTA GGTGACACTA TAGAATACGG AATTGTGAGC GGATAACAAT TCCA 54

SEQ ID NO:18
SEQUENCE LENGTH: 54
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
AGCTTGGAAT TGTTATCCGC TCACAATTCC GTATTCTATA GTGTCACCTA AATC 54

SEQ ID NO:19
SEQUENCE LENGTH: 20
SEQUENCE TYPE: amino acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: peptide
SEQUENCE DESCRIPTION:
Met Leu Pro Leu Asp Lys Asp Leu Gln Lys Ala Lys Ile Ser Ile
1 5 10 15
Thr Asp Phe Phe Glu
20

SEQ ID NO:20
SEQUENCE LENGTH: 23
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
FEATURE: 6, 9, 12 (inosine)
SEQUENCE DESCRIPTION:
ATGTTNCCNY TNGAYAARGA YYT 23

SEQ ID NO:21
SEQUENCE LENGTH: 23
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
FEATURE: 9 (inosine)
SEQUENCE DESCRIPTION:
AAGGATTTNC ARAARGCNAA RAT 23

SEQ ID NO:22
SEQUENCE LENGTH: 30
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
TAAATCTAAA CCAATTCCAA TAGATTGCTG 30

SEQ ID NO:23
SEQUENCE LENGTH: 30
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
CAAATCGGTT ACCAACGTGC TTTCTAGCAC 30

SEQ ID NO:24
SEQUENCE LENGTH: 30
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:
GAACTGTATT ACAAATTCCA TATGAAACTG 30

SEQ ID NO:25
SEQUENCE LENGTH: 30
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
GACAGAAAAT TTACAAATGC GAGAATGACC 30

SEQ ID NO:26
SEQUENCE LENGTH: 1588
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: Genomic DNA
SEQUENCE DESCRIPTION:
CCATGGCACA CGTTTCAAAA AAGAAATCCT CGAAGTCAAA TATGATGAGA AAAACATCTC 60
AGACATCCTG CATATGACGG TGGATGAAGC ATTGGAATTT TTCTCGGAAA ATCACGAAGA 120
AAAAATTGTA ACCAAACTAA AACCTTTGCA GGACGTTGGT TTGGGTTATC TTCAGTTAGG 180
CCAGTCCTCC TCTACTCTTT CCGGCGGTGA AGCCCAAAGA GTGAAGCTCG CCTCTTTCCT 240
TGTGAAAGGT GTAACGACGG AAAAAACGTT ATTTGTTTTT GATGAACCAT CAACAGGATT 300
ACATTTCCAC GACATTCAAA AATTACTGAA ATCACTTCAG GCACTGATAG AATTAGGGCA 360
TTCGGTTGTA GTGATTGAGC ATCAGCCGGA TATTATCAAA TGCGCCGATT ACATCATCGA 420
TGTCGGACCC AATGCCGGAA AATACGGTGG CGAAATTGTT TTCACAGGAA CTCCGGAAGA 480
TTTGGTAAAA GAGAAAAAGT CGTTTACAGG GAAGTATATT AAGGAGAAGT TAAAGTAATT 540
TATTTATATT TGAAGTTATG CTACCACTGG ATAAAGATTT ACAAAAAGCA AAGATTTCAA 600
TTACTGATTT TTTTGAAATT ACAAATAGAG TTTTAGATTA TTTCCCCAAT GTAATCAATA 660
ATACAGTTGA AAAAGGAGAT TATTTAATAT CCTCATCAAA TATTGCTGGA ACAATAAAAT 720
TCCTAAGACC AATCAATAGA AAGTTATTTA TTCAGGAAAA AAAAGTTTTC AATGATTATT 780
TTCAAAAACT GATTATAGTT TTTGAAAATA TAAGGAACAA AAAAACTGTA ACAGAGGAAG 840
ATAAAATTAT TATTGATAGG GTAATTTACA CAATACAGCA ATCTATTGGA ATTGGTTTAG 900

ATTTAATGGT TAATCAAAAT AGTGCTAGAA AGCACGTTGG TAACCGATTT GAAGAATTAA 960
TTAGAGTCAT TTTTACAGAA ATATCAGTAT CGAATAAAAG AACTGTATTA CAAATTCCAT 1020
ATGAAACTGA TGAAGGACAG AAAATTTACA AATGCGAGAA TGACCTCATT ATTTCTCCTT 1080
TTGAAAATGT AGAATCTACA AACAAACATC TAGATGAAAA TGAGATTGTT GTTTCAATAA 1140
AGACAACATC AAAAGATAGG ATGGGAAAAA TGTTTATAGA TAAAATTTTA CTTGAAAGGT 1200
TTGTTAAACA CCCTCAAAAA GTTATAGGGA TTTTCCTCAA TGATGTACAA AGAAAAGAAG 1260
ACAACAATAT CAGCTTTACA CTTGTTTCAG GATTATTTAT GGTGTATACT AAATTCTTAA 1320
CTACTCTTGA AGGGATCTAT TATTTAGATC CACCACCTAA TGCATTGAAA CTACCATATT 1380
CTAATCATAT GAAAAGATTT TCAGATTTAA TTACAGAAGA CCTTGAAAAA TTATTCTCCT 1440
CTTAATTTTT TTCTTCTATT TTGAGAATTT GAATCATCAT CGCCTGAAAA AATCCTGTTG 1500
ATCATACTTT CGTCTTTCAA TCGCTTTTGA ATTTCCAGAC AATAATTTTT ATCTAACTCT 1560
GTTCCAATCC ATTTTCTGCC AAAAGCTT 1588

SEQ ID NO:27
SEQUENCE LENGTH: 25
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Other nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
ATATTTGAAG CCATGGTACC ACTGG 25

SEQ ID NO:28
SEQUENCE LENGTH: 26
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: Oyther nucleic acid (synthetic DNA)
SEQUENCE DESCRIPTION:
GATGATTCAA ATTCTCACCA TGGAAG 26

SEQ ID NO:29
SEQUENCE LENGTH: 4146
SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: Genomic DNA
SEQUENCE DESCRIPTION:

AGATCTGGTC ATCCCAAACA AAAATCTTTC GGTTTACGAA GATGCAGTCG CTTCCTGGAA 60
AGGCGAAAGT ATGAGCGAAT GGAAAAAGGA ATTCATCAAA AAAGCCAAAG ATTTCCCAAT 120
TCACAAGCCT TATCATCAAC TCACAAAAGA GCAGAAACAG TTCCTTTGGA AAGGCGATAA 180
AACCAGAAGT TTCCCAAGTA TTGATAATTT TTTCAAAATG CTTGAAGAGA ATCTTTACAA 240
GATCCAATAC CGCGTAATGC TTTCGCGCTA TCGTGGGAAA ACACTTTGCC CCGATTGCGA 300
AGGATTACGA TTGCGGGAAG AAACAAGCTG GGTGAAGATT GACGGACACA ACATTCAGTC 360
TTTGATTGAA TTACCTTTGG ATGAACTCCT GCCATTGATC AAAAGCTTAA AACTGAACGT 420
CCACGACAGA GAAATTGCCA AACGCCTGAC TTACGAAATC GAAACGAGAT TAGAATTCCT 480
GACGAAAGTC GGCCTTGGAT ATCTGACTTT GAACCGAACA TCCAACACGC TTTCCGGAGG 540
AGAAAGCCAG AGAATCAATC TGGCGACAGC TTGGGAAGTT CGCTGGTTGG TTCTATTTAT 600
ATTTTGGATG AGCCGAGCAT TGGTCTGCAT TCCCGCGATA CAGAAAATCT GATTGGTGTC 660
CTCAAACAAC TCCGCGATTT GGGAANTACC GTGATTGTTG TAGAACACGA CGAAGATGTG 720
ATGCTTGCGG CAGNTTACAT TATAGATATT GGCCCNGNAG CGGGCTACCT TGGTGGCGAT 780
CTTGTTTTCA GCGNGGATTA TAAAGAGATG CTGAAGTNTN ATACTTTAAC CGCAAAATAC 840
CTGAATGGCG AACTGAAAAT AGAAGTTCCT GAAAAACGAA GAAAACCGAA GGAATTCATC 900
GCAATAAAAG GTGCCCGCCA GAATAATTTA AAAAATATTG ACGTTGATGT TCCGTTAGAA 960
TGTCTGACAG TTATCACAGG CGTTTCTGGA AGCGGGAAAT CCACTTTGAT GAAGGAAGTG 1020
ATGACCAATG CCATCCAGAT CCAACTGGGA ATGGGCGGCA AAAAAGCCGA TTACGATTCG 1080
GTGGAATTCC CGAAAAAGCT GATCCAGAAT ATCGAACTGA TTGACCAGAA CCCAATCGGG 1140
AAATCGTCCC GCTCCAACCC CGTGACTTAT CTGAAAGCTT ACGACGATAT CCGGGATCTT 1200
TTTGCGAAAC AAAAATCCGC AAAAATCCAG GGTTACAAAC CGAAGCATTT CTCCTTCAAT 1260
GTGGATGGCG GAAGATGTGA CGAGTGCAAA GGCGAAGGTA TCATTACCGT ATCAATGCAG 1320
TTTATGGCGG ACATCGAGCT GGAGTGTGAG CATTGCCATG GCACACGTTT CAAAAAAGAA 1380
ATCCTCGAAG TCAAATATGA TGAGAAAAAC ATCTCAGACA TCCTGCATAT GACGGTGGAT 1440
GAAGCATTGG AATTTTTCTC GGAAAATCAC GAAGAAAAAA TTGTAACCAA ACTAAAACCT 1500
TTGCAGGACG TTGGTTTGGG TTATCTTCAG TTAGGCCAGT CCTCCTCTAC TCTTTCCGGC 1560
GGTGAAGCCC AAAGAGTGAA GCTCGCCTCT TTCCTTGTGA AAGGTGTAAC GACGGAAAAA 1620
ACGTTATTTG TTTTTGATGA ACCATCAACA GGATTACATT TCCACGACAT TCAAAAATTA 1680
CTGAAATCAC TTCAGGCACT GATAGAATTA GGGCATTCGG TTGTAGTGAT TGAGCATCAG 1740
CCGGATATTA TCAAATGCGC CGATTACATC ATCGATGTCG GACCCAATGC CGGAAAATAC 1800

GGTGGCGAAA TTGTTTTCAC AGGAACTCCG GAAGATTTGG TAAAAGAGAA AAAGTCGTTT 1860
ACAGGGAAGT ATATTAAGGA GAAGTTAAAG TAATTTATTT ATATTTGAAG TTATGCTACC 1920
ACTGGATAAA GATTTACAAA AAGCAAAGAT TTCAATTACT GATTTTTTTG AAATTACAAA 1980
TAGAGTTTTA GATTATTTCC CCAATGTAAT CAATAATACA GTTGAAAAAG GAGATTATTT 2040
AATATCCTCA TCAAATATTG CTGGAACAAT AAAATTCCTA AGACCAATCA ATAGAAAGTT 2100
ATTTATTCAG GAAAAAAAAG TTTTCAATGA TTATTTTCAA AAACTGATTA TAGTTTTTGA 2160
AAATATAAGG AACAAAAAAA CTGTAACAGA GGAAGATAAA ATTATTATTG ATAGGGTAAT 2220
TTACACAATA CAGCAATCTA TTGGAATTGG TTTAGATTTA ATGGTTAATC AAAATAGTGC 2280
TAGAAAGCAC GTTGGTAACC GATTTGAAGA ATTAATTAGA GTCATTTTTA CAGAAATATC 2340
AGTATCGAAT AAAAGAACTG TATTACAAAT TCCATATGAA ACTGATGAAG GACAGAAAAT 2400
TTACAAATGC GAGAATGACC TCATTATTTC TCCTTTTGAA AATGTAGAAT CTACAAACAA 2460
ACATCTAGAT GAAAATGAGA TTGTTGTTTC AATAAAGACA ACATCAAAAG ATAGGATGGG 2520
AAAAATGTTT ATAGATAAAA TTTTACTTGA AAGGTTTGTT AAACACCCTC AAAAAGTTAT 2580
AGGGATTTTC CTCAATGATG TACAAAGAAA AGAAGACAAC AATATCAGCT TTACACTTGT 2640
TTCAGGATTA TTTATGGTGT ATACTAAATT CTTAACTACT CTTGAAGGGA TCTATTATTT 2700
AGATCCACCA CCTAATGCAT TGAAACTACC ATATTCTAAT CATATGAAAA GATTTTCAGA 2760
TTTAATTACA GAAGACCTTG AAAAATTATT CTCCTCTTAA TTTTTTTCTT CTATTTTGAG 2820
AATTTGAATC ATCATCGCCT GAAAAAATCC TGTTGATCAT ACTTTCGTCT TTCAATCGCT 2880
TTTGAATTTC CAGACAATAA TTTTTATCTA ACTCTGTTCC AATCCATTTT CTGCCAAAAG 2940
CTTCCGCAAC AGCATATGTA GTTCCCGAAC CACCAAATGG ATCAAGAATT AAACTATCTT 3000
TATTAGATGA AGATAAAATT ATTTTTTTAA GCAATTCTAC TGGCTTTTGC GTAGGGTGAA 3060
TACTTCTTTC CCAAGAACCA TTTGAAATTG TGGGAATCTC CAAAACATCT TTTGGCTTAG 3120
CTCCTAATGG GTTTGGCTCC CAAATATATT GCTTTTTTGA GTTCGAATAT TGCGAAGATT 3180
CGGCCTGGGT TCTTTGTGGA TATTTAACTG TATGCTCATT ATACGGGATT CGTGCCTCAT 3240
CAATATTAAA AATAAAATTT TTAGATTTTC TTAATAACAG TATACTTTCG TGTGAACGTC 3300
CCCAATCTTT ACCTAAATTT GCCTTGTTTC TATAGAACCA AATCAACCAT TTACAACTGT 3360
GAAAATATTT TGAAGTGATA AATTTTATGT CTGCCAGAAT TTCTGAAAAG CCACAAACAT 3420
ATAAACTGCC ATTATCTTTT AAAACTCTAT GAGCTTCCAT TATCCATTCT TTACTCCATT 3480
CGAGATATTC ATTTTGAGAA GAAAAAATAT CCCACTCTGC CTTTTTAATG TTATAAGGTG 3540
GATCTGCAAA AATTAAATCC ACAGAATTAC TCTCTAATTT TCTTAAAAAA TCAATAGCAT 3600
CAACATTGAA AAGTGAACCG TTTTTTGAAA TAAAAAATGG TTTAAGCTCG ATTTCCGAAT 3660
AAAGTTTTAA TTTTGGTATA TAACTGTAAT TATCAAACGC TATTTCATTC ACAAATGAAT 3720
CAATCTGCTG TTGTGTATAA ACCCTGTAAT TATTAATAGG ATGTCTTAAA CTTTTGAATT 3780
TTCCAGAATT ATCCCATCTT CCTTAATGTC TCAGAGTTAA CATCTAATAA TTTCGCCGCT 3840

TCTTTTATTG ATAAATAATC ATCCATATCT TACACAACAT TACACAAGTT TATACAGCAA 3900
ATATAAATAT TTTTTATACA TTGTAAAAAT TTTATTTACT TTTATTTTGT TCAATTGTCT 3960
CAATAAATAG TTAATCGAAA TACATTTTGA ATATGATAAA ATTGACTCCA ACAAATCTAA 4020
CACAATGACA TTAAAACCAA TAAAAACGGA AGAAGATTAC AATCAGGTTT TAGAAAGACT 4080
TTCACAAATT TTCGACGCTA AACCAAATAC CAAAGATGGA GATGAATTGG GAAATCTTGG 4140
GAATTC 4146

SEQ ID NO:30
SEQUENCE LENGTH: 23
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: Genomic DNA
SEQUENCE DESCRIPTION:
ATTTAGGTGA CACTATAGAA TAC 23